

SULFITE OXIDASE FROM CHICKEN LIVER

The role of carboxyl and imidazole groups
in the reaction with cytochrome c

INAUGURAL-DISSERTATION

zur Erlangung der Philosophischen Doktorwürde

vorgelegt der

Philosophischen Fakultät II

der

Universität Zürich

von

MARTIN RITZMANN

von Osterfingen SH

Begutachtet von Herrn Prof. Dr. H.R. Bosshard

Zürich 1987

Zentralstelle der Studentenschaft



SULFITE OXIDASE FROM CHICKEN LIVER

The role of carboxyl and imidazole groups
in the reaction with cytochrome c

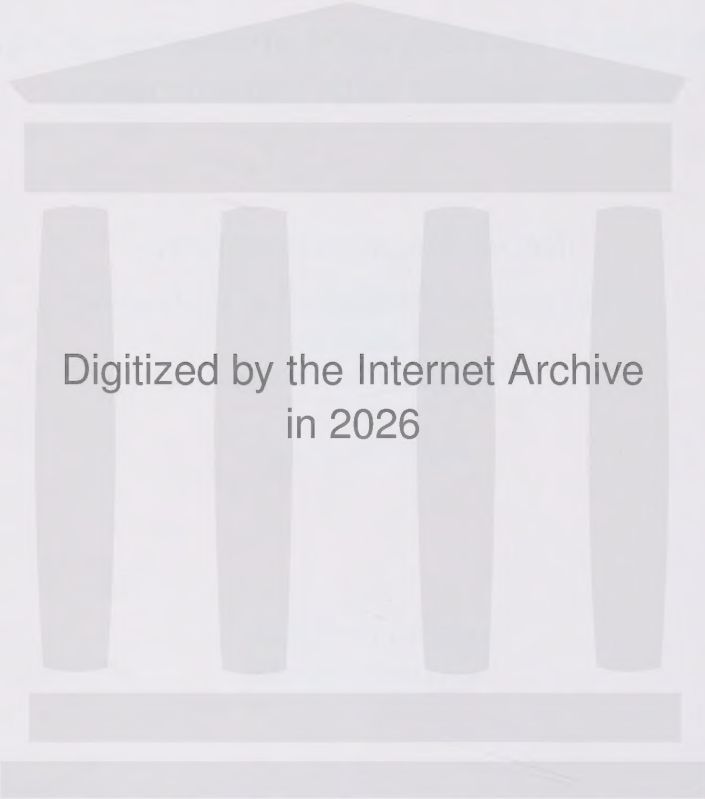
INAUGURAL-DISSERTATION

zur Erlangung der Philosophischen Doktorwürde
vorgelegt der
Philosophischen Fakultät II
der
Universität Zürich

von
MARTIN RITZMANN
von Osterfingen SH

Begutachtet von Herrn Prof. Dr. H.R. Bosshard

Zürich 1987
Zentralstelle der Studentenschaft



Digitized by the Internet Archive
in 2026

DANK

An dieser Stelle möchte ich allen danken, die zum Gelingen dieser Arbeit in irgend einer Weise beigetragen haben, sei es durch praktische Hilfeleistung, durch hilfreiche Diskussionen oder auch durch Anteilnahme in guten und weniger guten Zeiten.

Besonders bedanken möchte ich mich bei Herrn Prof. Dr. H.R. Bosshard für seine Ideen und Anregungen und für seine Unterstützung in den verschiedenen Phasen meiner Arbeit. Danken möchte ich auch Bruno Michel, für seine Mithilfe bei der Bestimmung der kinetischen Konstanten modifizierter Sulfitoxidase und bei der Herstellung der Figuren.

Allen Mitarbeitern der Forschungsgruppe von Prof. Dr. H.R. Bosshard danke ich herzlich für die vielfältige Hilfe bei allen Problemen, die Einführung in neue Techniken, für alle Anteilnahme und Aufmunterung und für die gute Atmosphäre in unseren Labors.

CONTENTS

1. INTRODUCTION

1.1. Sulfur in animal systems	1
1.1.1. Origin and health effects	1
1.1.2. Detoxification: The role of sulfite oxidase	2
1.2. Properties of sulfite oxidase	4
1.2.1. Molecular weight, spectral properties and prosthetic groups	4
1.2.2. Domain structure	7
1.2.3. Distribution and localisation	9
1.2.4. Reaction mechanism	9
1.2.5. Sequence and structure	11
1.2.6. The sulfite oxidase-cytochrome c electron transfer complex	12
1.3. Aim of the present study	15

2. MATERIAL AND METHODS

2.1. Biochemical methods	16
2.1.1. Chicken liver sulfite oxidase	16
2.1.1.1. Isolation procedure	16
2.1.1.2. Enzyme assays	17
2.1.2. Isolation of the domains of sulfite oxidase	18
2.1.2.1. Cleavage by chymotrypsin	18
2.1.2.2. Cleavage by subtilisin	18
2.1.3. Binding of cytochrome c to sulfite oxidase	19
2.1.4. Immunological methods	19
2.1.4.1. Production of antisera	19
2.1.4.2. Ouchterlony double diffusion	20
2.2. Chemical modification of sulfite oxidase	21
2.2.1. Modification of carboxyl groups	21
2.2.1.1. Reaction with Woodward's reagent K	21
2.2.1.2. Synthesis of N-cyclohexyl-N'-(2-(N-[³ H]methylmorpholino)ethyl)carbodiimide p-toluene sulfonate	21
2.2.1.3. Reaction of sulfite oxidase with carbodiimides	22
2.2.2. Modification of histidine residues	25

3. RESULTS	
3.1. Isolation and storage of chicken liver sulfite oxidase	26
3.2. Cleavage of sulfite oxidase by subtilisin	29
3.3. Binding of cytochrome c to sulfite oxidase	32
3.4. Rabbit-anti-chicken sulfite oxidase antisera	33
3.5. Modification of carboxyl groups with Woodward's reagent K	36
3.6. Modification of carboxyl groups by carbodiimides	37
3.6.1. Selectivity of the reagent	37
3.6.2. Effect of cytochrome c	40
3.6.3. Effect of nucleophiles	42
3.6.4. Kinetics of inactivation of sulfite oxidase by CMC	44
3.6.5. Incorporation of [³ H]-CMC and its effect on activity and kinetic constants of sulfite oxidase	46
3.7. Modification of histidine residues by DEP	52
3.7.1. Selectivity of the reagent	52
3.7.2. Effect of cytochrome c	54
3.7.3. Incorporation of DEP and its influence on activity and kinetic constants of sulfite oxidase	55
4. DISCUSSION	
4.1. Binding of cytochrome c to sulfite oxidase	60
4.2. The reaction of sulfite oxidase with cytochrome c	61
4.3. The role of carboxyl groups for the enzymatic activity of sulfite oxidase	63
4.4. The role of histidine residues for the enzymatic activity of sulfite oxidase	67
4.5. Conclusions	70
REFERENCES	72
ABBREVIATIONS AND TRIVIAL NAMES	86
SUMMARY	87
ZUSAMMENFASSUNG	89

1. INTRODUCTION

1.1. SULFUR IN ANIMAL SYSTEMS

1.1.1. Origin and Health Effects

Sulfur dioxide is unique among environmental substances because of the many routes of human exposure to it. Combustion of coal and oil releases SO_2 into urban atmospheres. It is added to foods, beverages, and pharmaceuticals and we produce it within our bodies as a product of the catabolism of sulfur containing amino acids.

Respiratory abnormalities have been most thoroughly investigated among the health effects induced by chronic levels of SO_2 . Various animal studies have revealed that elevated levels of SO_2 exposure cause the development of narrowed air passages, impairment of normal pulmonary mechanics, thickening of alveoli, lymphatic engorgement, edema, alveolar hyperplasia, and the acceleration of the onset of precancerous conditions and lung tumors in susceptible mice (Admur, 1971; Balchum, 1959; Alarie et al., 1972; Alarie et al., 1975; Wakisaka, 1976).

Sulfite has been used in the preparation of wine since ancient time. It is currently used as a preservative in a variety of substances, including beer, preserved fruits and vegetables, fruit juices and syrups.

Sulfur oxides may enter the body by inhaling SO_2 or by consuming foods treated with sulfite or bisulfite salts. SO_2 is absorbed to the bloodstream to form sulfite (SO_3^{2-}) or bisulfite (HSO_3^-). Once in the blood the bisulfite is distributed to all body tissues including the brain (Hickey et al., 1974).

Bisulfite is known to react with numerous biomolecules, essentially acting as nucleophile attracted to electrophilic centers. Exposure of laboratory animals to $^{35}\text{SO}_2$ has revealed that much of the bisulfite reacts with the disulfide bonds of plasma proteins, alpha-globulin and albumin

to form S-sulfonates (Yokoyama et al., 1971):



Reaction with other compounds seems to be much less important.

Several studies have indicated that SO_2 exposure may interfere with normal immunological mechanisms, especially those concerning the removal of microorganisms and the production of antibodies and agglutinins (reviewed by Calabrese et al., 1981).

It is known that bisulfite ions can selectively deplete red blood cells of 2,3-diphosphoglycerate as shown by Parker (1969). Therefore, bisulfite may potentially deplete the body tissues of oxygen.

A most serious property of SO_2 is its mutagenic capability. While the deamination of cytosine by 0.5 M or higher concentrations of bisulfite, at pH 5.6, has been a useful biochemical and genetic tool (Shapiro et al., 1973), it is unlikely to be important with respect to human health. The above conditions are far from physiological ones. However, various genetic effects, including mutations, have been observed at lower bisulfite concentrations and at neutral pH in other systems. These effects are presumably caused by reactions other than the deamination of cytosine. The results of these studies are reviewed by Shapiro (1977) and Gunnison et al. (1981).

The number of severe effects caused by sulfite demonstrates the importance of a potent detoxification system to protect the organism from this threat.

1.1.2. Detoxification: The Role of Sulfite Oxidase

Bisulfite is produced endogenously in mammals, as an intermediate in the catabolism of sulfur containing amino acids. The amount of bisulfite produced in this manner undoubtedly exceeds the quantity absorbed from the environment (Shapiro, 1983). The terminal step in the process of amino acid catabolism is the oxidation of bisulfite to sulfate.

In 1922 Schmid & Clark reported that administration of Na_2SO_3 resulted in the rapid *in vivo* oxidation to inorganic sulfate in dog and man, but it was not until 1953 (Heimberg et al., 1953) that the reaction was shown to be enzyme catalyzed. Since then the enzymatic oxidation of sulfite to sulfate has been demonstrated in animal tissues (MacLeod et al., 1961; Mudd et al., 1967; Joshi et al., 1969; Kessler & Rajagopalan, 1972; Gibson & Strong, 1973) as well as in plants (Tager & Rautanen, 1955; Arrigoni, 1959; Fromageot et al., 1960) and in certain bacteria (Charles & Suzuki, 1966; Charles, 1969; Lyric & Suzuki, 1970).

The enzyme catalyzing the conversion of sulfite to sulfate has been termed sulfite oxidase. That no other mechanism of sulfite oxidation is of physiological relevance in man was definitely established by the discovery by Mudd and coworkers of a case of apparent sulfite oxidase deficiency in an infant with symptoms of neurological abnormalities, mental retardation and severe metabolic disturbances (Mudd et al., 1967; Irreverre et al., 1967; Percy et al., 1968). Post-mortem examination of various tissues indeed showed no detectable sulfite oxidase activity in kidney, liver and brain. Elevated levels of S-sulfo-L-cysteine, sulfite, and thio-sulfate were found in the deficient patient's urine while no inorganic sulfate could be detected.

Two other cases of sulfite oxidase deficiency have been described since 1967 by Shih et al. (1977) and by Duran et al. (1979). It has been postulated by Olney et al. (1975) that S-sulfocysteine may be the neurotoxic agent responsible for brain damage in sulfite oxidase deficiency.

In the absence of sulfite oxidase activity excess sulfite may be converted to thiosulfate via the action of beta-mercaptopyruvate sulfurtransferase (Segal & Johnson, 1963) and possibly to S-sulfocysteine via glutathione as proposed by Kagedal et al. (1986).

Total sulfite oxidase deficiency in human is thought to be extremely rare. But it is hypothesized that individuals with a intermediate deficiency

(heterozygotes) are at increased risk to the mutagenic and other potential health effects of SO_2 exposure (Calabrese et al., 1981).

The importance of sulfite oxidase in detoxification is further supported by the inverse correlation found between the levels of sulfite oxidase activity in the liver and kidney and bisulfite toxicity in five species of laboratory animals (Tejnarova, 1977; Tejnarova, 1978).

Since sulfite oxidase contains molybdenum as a cofactor, sulfite oxidase deficiency could be produced in rats by supplying a low molybdenum diet and concomitantly administering tungsten, a competitive antagonist of molybdenum. The decrease in activity of sulfite oxidase and xanthine oxidase, the only other molybdenum-containing enzyme in rats, was dose-dependent with a parallel loss of hepatic molybdenum (Cohen et al., 1973). No physical abnormalities were observed except the decreased enzyme activities. The LD_{50} for bisulfite however was between 148 and 221 mg NaHSO_3 /kg of body weight of the tungsten treated rats, compared to 394 - 569 mg/kg for rats fed with a normal diet. Similarly, sulfite oxidase deficient rats exposed to 925 and 2320 ppm SO_2 exhibited significant decreases in survival times as compared to the appropriate controls at the respective concentrations (Johnson & Rajagopalan, 1974).

All these findings support the very important role of sulfite oxidase in detoxification of sulfite.

1.2. PROPERTIES OF SULFITE OXIDASE

1.2.1. Molecular Weight, Spectral Properties and Prosthetic Groups

Sedimentation equilibrium centrifugation studies established a molecular weight of 115,000 for bovine sulfite oxidase (Cohen & Fridovich, 1971a), while SDS-polyacrylamide gel electrophoresis in the presence or absence of mercaptoethanol revealed a single protein band of 55,000 molecular weight. These data indicate that native sulfite oxidase is a non-covalent dimer of subunits of identical size.

Native molecular weights of 108,000 and 120,000 (and subunit molecular weights of 55,000 and 57,200) were obtained for the chicken (Kessler & Rajagopalan, 1972) and rat (Southerland et al., 1978) sulfite oxidase, respectively. Polyacrylamide gel electrophoresis in the presence of SDS indicated a subunit molecular weight of 61,000 for the human enzyme (Johnson & Rajagopalan, 1976), the highest value among those characterized so far. Sulfite oxidase proteins from other sources have similar structural properties. The only apparent exception is the enzyme from *Thiobacillus thioparus* for which a native molecular weight of about 54,000 has been reported (Lyric & Suzuki, 1970).

The first partial purification of sulfite oxidase was performed by MacLeod et al. (1961). They reported the enzyme to be a hemoprotein. The absorption spectrum of the reduced enzyme resembles that of cytochrome b_5 .

Definitive identification of sulfite oxidase as a hemoprotein was reported by Cohen and Fridovich (1971a). They noted for the bovine enzyme a parallel enrichment of activity and heme content, coincidence of heme staining and activity staining bands after polyacrylamide gel electrophoresis, and reducibility of the heme by sulfite. The reduced spectrum was strikingly similar to that of reduced microsomal cytochrome b_5 . The non-covalently bound heme was identified as protoporphyrin IX based on its pyridine hemochromogen. The heme content was estimated to be one mol per enzyme monomer.

The absorbance spectra of oxidized and reduced sulfite oxidase of chicken liver are shown in figure 1. The absorption maximum for the oxidized enzyme was at 413 nm, with an extinction coefficient of $113 \text{ mM}^{-1}\text{cm}^{-1}$, and for the reduced enzyme at 424 nm, with $172 \text{ mM}^{-1}\text{cm}^{-1}$ (Rajagopalan, 1980). Complete reduction occurs with sulfite, since addition of sodium dithionite has no further effect on the sulfite reduced sample.

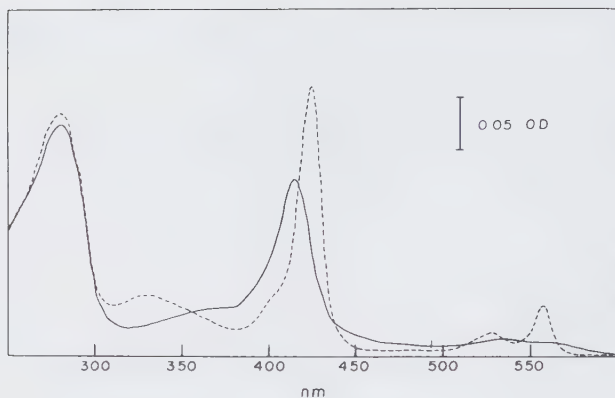


Figure 1: Absorption spectra of chicken liver sulfite oxidase

The spectra of the oxidized enzyme (————) and of the sulfite-reduced enzyme (-----) is depicted, as shown by Rajagopalan (1980).

The second prosthetic group of the enzyme, the molybdenum-cofactor, was discovered quite by accident as the result of a casual examination of a partially purified enzyme by EPR spectroscopy (Cohen et al., 1971). While the oxidized enzyme failed to show any EPR signal at -180°C , enzyme reduced with sulfite showed a strong signal at $g = 1.97$ (figure 2).

The presence of molybdenum in the enzyme was confirmed by colorimetric analysis which yielded a value of one atom of molybdenum per enzyme monomer and a molybdenum to heme ratio of 1.05. A structure of the molybdenum-cofactor was proposed by Johnson & Rajagopalan (1982). They proposed urothione, a sulfur-containing pterin, characterized by Goto et al. (1969) to be the end product of the degradation of the cofactor. The finding that urine samples from patients deficient in the molybdenum-cofactor are devoid of urothione demonstrates a metabolic link between the two molecules.

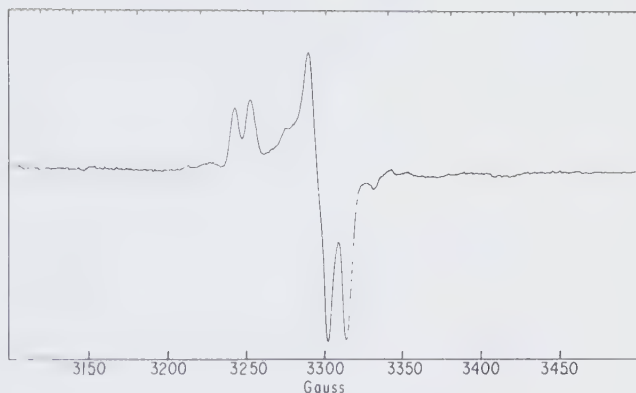


Figure 2: EPR spectrum of sulfite-reduced sulfite oxidase
The figure depicts the spectrum of the bovine liver enzyme in 0.1 M TrisHCl, pH 7.0 as shown by Cohen et al. (1971).

1.2.2. Domain Structure

The possibility that proteolytic treatment of sulfite oxidase could lead to partial inactivation was first suggested by MacLeod et al. (1961) who found that exposure of the bovine enzyme to trypsin led to 90 % inhibition of cytochrome c reduction. A similar effect was shown for the rat enzyme by Johnson & Rajagopalan (1977). Gel filtration of the proteolytic product showed two major fragments of very different size. The smaller fragment has a molecular weight of 9,500 and appears to be monomeric. It contains the heme, no sulfite oxidase activity and is only reducible by dithionite. This fragment is called heme-domain.

The large fragment has a molecular weight of 47,400 under denaturing conditions, but exists as a dimer in the native form. It contains no heme, but all of the molybdenum and retained the ferricyanide and the O_2 reductase activity of the enzyme, but lacks the cytochrome c reductase

activity. The heme-domain, which was found to be at the NH_2 -terminus of the enzyme, is therefore required for the electron transfer to the physiological acceptor cytochrome c. The pathway of electrons from sulfite to three possible electron acceptors is shown in figure 3.

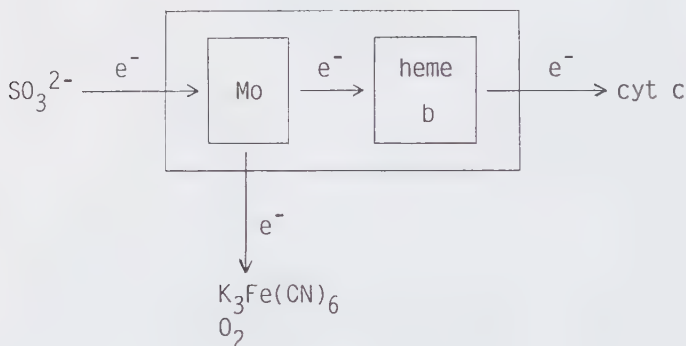


Figure 3: Electron transfer by sulfite oxidase

The figure shows the pathways of electrons from sulfite to three possible electron acceptors of which cytochrome c is the physiological one.

Proteolysis of the enzyme with papain and chymotrypsin was performed by Southerland et al. (1978). The size differences of the heme- and molybdenum-domains obtained by different proteases suggested that the hinge segment between the two domains is at least 30 residues in length. This interdomain hinge region has been shown to be less accessible to proteolytic enzymes when sulfite oxidase is in the reduced form (Southerland & Rajagopalan, 1978).

Surprisingly, the behavior of the chicken liver enzyme was quite different. As shown by Guiard & Lederer (1977) it was not possible to obtain two discrete domains by treatment of the enzyme with proteases. With chymotrypsin it was at least possible to obtain the intact heme-domain.

1.2.3. Distribution and Localization

Sulfite oxidase seems to be present in nearly all living organisms, but its distribution in the different tissues of an organism is very irregular. MacLeod et al. (1961) found high levels of the enzyme in rat liver, kidney, and heart and lower levels in spleen, testes, brain, and lung. No activity was found in rat blood (Johnson et al., 1977).

The definite studies of Wattiaux-De Coninck & Wattiaux (1971) resolved the earlier uncertainty regarding the subcellular localization of sulfite oxidase and established the mitochondrial origin of the enzyme in the rat liver. They further showed that the pattern of extractability of sulfite oxidase from mitochondria was identical to that of adenylate kinase, an enzyme which had earlier been shown to be a marker for the inter-membrane space of mitochondria (Novikoff et al., 1952). The conclusion that sulfite oxidase is localized in the inter-membrane space of mitochondria was corroborated by the work of Fukushima et al. (1972) who identified the same mitochondrial compartment as the milieu of a "large cytochrome b_5 " of 100.000 molecular weight, which was shown to be sulfite oxidase (Ito, 1971).

1.2.4. Reaction Mechanism

Sulfite oxidase is extremely specific for sulfite as the electron donor. Sulfite can not be replaced by NADH, NADPH, succinate, thiosulfate, dithionate, cysteine sulfinic acid, cysteine thiosulfate, lactate, acetaldehyde, hypoxanthine, selenite (MacLeod et al., 1961), nitrate, or xanthine (Cohen & Fridovich, 1971). But the enzyme can use a wide variety of electron acceptors such as cytochrome c, ferricyanide, O_2 , 2,6-dichloroindophenol and methylene blue (Cohen & Fridovich, 1971). The physiological electron acceptor was found to be ferricytochrome c (Rajagopalan, 1980). The physiological reaction can be described by the equation



The conversion of sulfite to sulfate is a two electron oxidation, whereas cytochrome c is a one electron acceptor. Since there is no evidence for the formation of a sulfite radical in the process of oxidation by sulfite oxidase it appears reasonable to conclude that the oxidation step occurs by a two electron step.

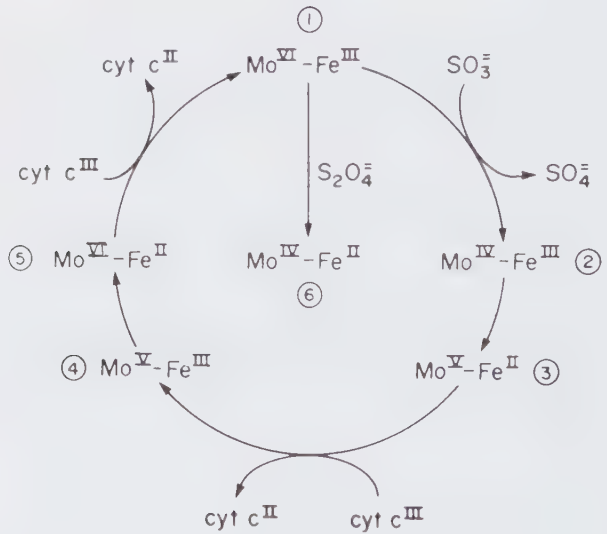


Figure 4: The proposed catalytic cycle for sulfite oxidase
The figure shows the catalytic cycle proposed by Rajagopalan (1980).

A proposed oxidation-reduction cycle (Rajagopalan, 1980) is shown in figure 4. The oxidized resting enzyme (1) contains molybdenum VI and ferric iron. Two electron reduction by a molecule of sulfite yields species (2) with molybdenum in the IV stage. Rapid transfer of one electron from molybdenum to heme produces an enzyme (3) poised for interaction by cytochrome c. When the ferrous iron in the enzyme molecule has been oxidized by cytochrome c, the second electron can move from the molybdenum to the enzyme heme (4 - 5), and an other molecule of cytochrome c can be reduced. Complete reduction of the electron carriers in sulfite oxidase can be achieved by dithionite, yielding species (6).

1.2.5. Sequence and Structure

The information available about the sequence and structure of sulfite oxidase is limited. The heme-domain of the rat (Rajagopalan, 1980) and the chicken liver enzyme (Guiard & Lederer, 1979) have been sequenced. The amino acid sequence of the chicken heme-domain was found to be homologous to that of microsomal cytochrome b_5 . Inspection of the calf liver cytochrome b_5 three-dimensional model (Mathews et al., 1972) together with the alignment of the sequences of the two proteins suggests a similar overall structure for sulfite oxidase heme-domain with a limited number of backbone modifications as shown in figure 5 (Guiard & Lederer, 1979).

Sulfite oxidase, microsomal cytochrome b_5 and baker's yeast flavocytochrome b_2 together constitute a " b_5 -superfamily" (Guiard & Lederer, 1979a) with a similar binding site for cytochrome c. In contrast, there is no obvious sequence homology with cytochrome c peroxidase, with subunit II of cytochrome c oxidase, or with cytochrome c_1 of cytochrome reductase, even though these proteins share with sulfite oxidase, cytochrome b_5 and flavocytochrome b_2 the property of using cytochrome c as a common physiological redox partner.

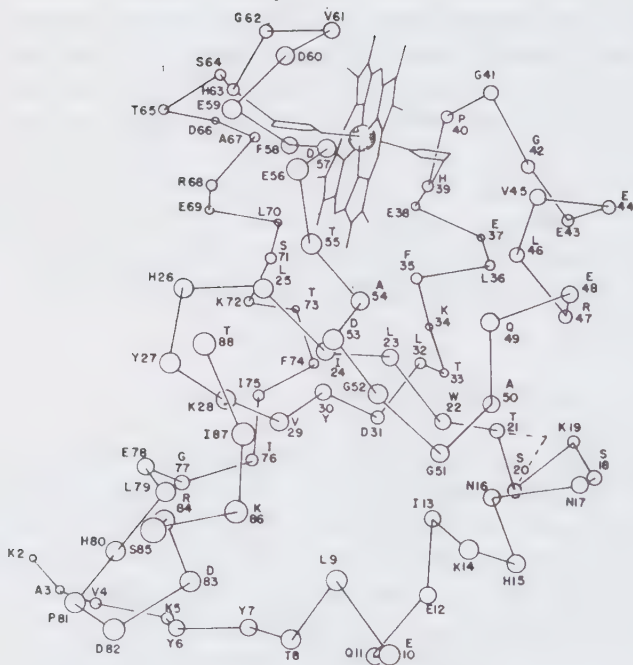


Figure 5: The proposed structure of the sulfite oxidase heme-domain
The figure shows the structure of the heme-domain of chicken liver sulfite oxidase as proposed by Guiard & Lederer (1979) according to the three-dimensional model of calf liver cytochrome b_5 by Mathews et al. (1972). Dashed lines indicate the proposed deletion and insertions.

1.2.6. The Sulfite Oxidase-Cytochrome c Electron Transfer Complex

The lack of sequence homology between sulfite oxidase and other enzymes that use cytochrome c as a common physiological redox partner is surprising, since during electron transfer, the different proteins recognize the

very same surface area of the cytochrome c molecule (Webb et al., 1980; Speck et al., 1979; Smith et al., 1980; Speck et al., 1981; Rieder & Bosshard, 1980; Margoliash & Bosshard, 1983).

Figure 6 shows a schematic representation of the binding domains on cytochrome c for four different enzymes (Koppenol & Margoliash, 1982). It is assumed that all of the physiological redox partners share some topological features that must be necessary for proper alignment with the conserved electron transfer interaction domain of cytochrome c (Margoliash & Bosshard, 1983).

Salemme (1976) proposed a model for the interaction between cytochrome b_5 and cytochrome c based on a steric complementarity between the two molecules. The complex was stabilized by Van der Waals' interactions and allowed for charge pairing between four negative groups on cytochrome b_5 and four conserved positive ones on cytochrome c. But only two of these negative groups are conserved in sulfite oxidase. In the absence of actual three-dimensional evidence, it cannot be ruled out that other negative charges in the vicinity of the non-conserved ones might replace them. Inspection of the sequences and of the molecular model, however, does not provide obvious support for this possibility at present.

In the case of cytochrome c peroxidase from yeast the molecular surface recognized by cytochrome c has been mapped in considerable detail. Based on computer-aided model building electron transfer complexes with cytochrome c were proposed (Poulos & Kraut, 1980; Finzel et al., 1984). The interaction is governed by ionic bonds between carboxylate groups of the peroxidase and protonated lysyl side chains of cytochrome c (Waldmeyer & Bosshard, 1985; Bechtold & Bosshard, 1985; Poulos & Kraut, 1980).

A single histidine residue of cytochrome c peroxidase is thought to be crucial for activity (Bosshard et al., 1984; Finzel et al., 1984).

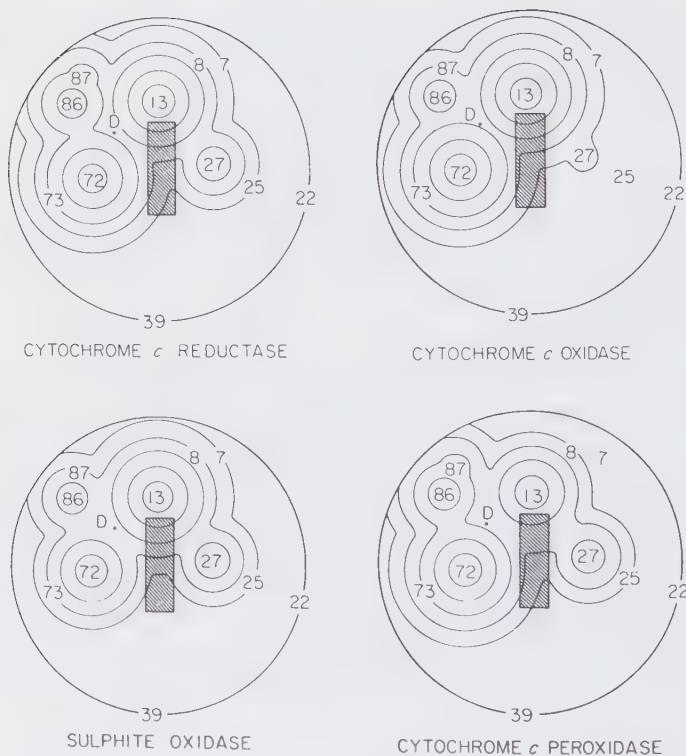


Figure 6: Schematic representation of the domain of cytochrome c for interaction with four of its redox partners

The figure shows the interaction domain on cytochrome c for cytochrome c reductase, cytochrome c oxidase, sulfite oxidase, and cytochrome c peroxidase as depicted by Koppenol & Margoliash (1982). The numbers refer to the sequence position of lysine residues that were shown to be located at the intermolecular contact site in the four different electron transfer complexes. The hatched bar symbolizes the heme edge facing the viewer.

1. 3. AIM OF THE PRESENT STUDY

The binding site on cytochrome c for its different reaction partners has been mapped in considerable detail. The same set of lysines has been found to be important for proper binding to all reaction partners.

In the case of cytochrome c oxidase (Millet et al., 1983) and cytochrome c peroxidase (Bechtold & Bosshard, 1985, Waldmeyer & Bosshard, 1985) a corresponding set of carboxylate groups has been found that interacts with particular lysines of cytochrome c. Surprisingly, there is no sequence homology between these two enzymes and sulfite oxidase which also uses cytochrome c as its physiological electron acceptor.

The main aim of the present work was therefore to see if, despite the lack of structural homologues, the mode of interaction between sulfite oxidase and cytochrome c follows the principles that govern the interaction with cytochrome c oxidase and cytochrome c peroxidase. In particular, the role of carboxylate and imidazole groups in binding and electron transfer should be clarified. To this end, binding of horse cytochrome c to chicken liver sulfite oxidase was examined by gel filtration. Chemical modification of carboxyl and imidazole groups of the enzyme was performed in presence and absence of cytochrome c and its influence on the steady state assay and on the kinetic constants of the enzymatic reaction was investigated.

A goal beyond the limited context of this dissertation will be the detailed elucidation of the electron transfer interaction domain of sulfite oxidase for cytochrome c.

2. MATERIALS AND METHODS

2.1. BIOCHEMICAL METHODS

2.1.1. Chicken Liver Sulfite Oxidase

2.1.1.1. Isolation Procedure

Sulfite oxidase was prepared from chicken liver, either fresh or frozen immediately after slaughtering. The livers were obtained from local supermarkets. An acetone powder was prepared according to Cohen and Fridovich (1971) and the enzyme purified as described by Johnson and Rajagopalan (1976), except that the final gel filtration step was replaced by affinity chromatography on a column of Sepharose 4B coupled with cytochrome c by the following procedure:

Seven g of CNBr-activated Sepharose 4B (Pharmacia) was swollen for 15 minutes at room temperature in 1 mM HCl and washed extensively on a sintered glass filter with the same solution. The gel was then washed once with the coupling buffer (166 mM sodium borate, 134 mM NaCl, pH adjusted to 8.2 with NaOH) and was immediately transferred to 50 ml of a solution of 75 mg cytochrome c (Sigma, type III) in the coupling buffer. The mixture was shaken for 3 hours at room temperature. Fifty ml of 1 M ethanolamine-HCl, pH 8.2, was added to block remaining active groups and the mixture was shaken for another 3 hours. The gel was then washed with 5 cycles of coupling buffer and 0.1 M acetic acid, 0.5 M NaCl, pH 8.2. It was stored in the coupling buffer at 4°C.

Fourteen ml of the resin was packed into a column (0.9 x 22 cm) and equilibrated overnight with starting buffer (5 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM PMSF, pH 7.8). The pooled fractions after DE-32 anion exchange chromatography containing the partially purified enzyme (Johnson & Rajagopalan, 1976) were equilibrated with starting buffer by concentrating and diluting three times by ultrafiltration in a Diaflo equipped with an UF C-50 membrane (Nucleopore). The sample (about 5 ml) was

loaded onto the column, washed with 30 ml of starting buffer and eluted with a linear gradient from 5 mM to 100 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM PMSF, pH 7.8 (2 x 150 ml) at a flow rate of 7 ml/h. Fractions with a A_{413} / A_{280} ratio of at least 0.80 were pooled as pool A, those with a ratio of at least 0.75 as pool B. The specific activity was always above 6000 U/mg in the cytochrome c assay. The purified enzyme was stored in aliquots at -70°C as eluted from the affinity column.

The concentration of the enzyme was determined from A_{413} ($\Delta\epsilon = 113 \text{ mM}^{-1}\text{cm}^{-1}$) for the oxidized enzyme or, after reduction with sulfite or dithionite, from A_{424} ($\Delta\epsilon = 172 \text{ mM}^{-1}\text{cm}^{-1}$), as described by Rajagopalan (1980).

Polyacrylamide gel electrophoresis of the enzyme in the presence of sodium dodecylsulfate (SDS) according to Lämmlí (1970) gave a single band plus some minor impurities that accounted for 10% or less of the total Coomassie blue stainable protein.

Protein concentrations were measured with the Coomassie blue protein assay of Bradford (1976), using the reagent supplied by Bio-Rad. Bovine serum albumin was used as standard.

2.1.1.2. Enzyme Assays

Assays with cytochrome c or with ferricyanide as electron acceptors were performed essentially as described by Cohen & Fridovich (1971). For the cytochrome c assay the reduction was followed at 550 nm in a solution containing in a total volume of 2.00 ml, 1-10 μl of appropriately diluted enzyme, 0.4 mM sodium sulfite (Fluka puriss.), 10 μM ferricytochrome c, 0.1 mM EDTA, and 0.1 M Tris-HCl, pH 7.5. The ferricyanide assay was conducted under the same conditions, using ferricyanide (0.4 mM, Merck, p.a.) instead of ferricytochrome c. One unit of activity was defined as a change of 0.1 absorption unit per minute. Assays were performed at 20°C .

2.1.2. Isolation of the Domains of Sulfite Oxidase

2.1.2.1. Cleavage by Chymotrypsin

The digestion was performed as described by Gulard and Lederer (1977). Sulfite oxidase ($60\text{ }\mu\text{M}$) in 0.1 mM potassium phosphate, pH 8.0 was incubated with chymotrypsin (Boehringer, A_4) ($1/20$; w/w) at 37°C . After 9 hours 0.1 M PMSF in isopropanol was added to a final concentration of 1 mM . The reaction mixture was applied on a Bio-Gel P-60, fine, gel filtration column ($2.6 \times 100\text{ cm}$, Bio Rad) and eluted with 0.1 M potassium phosphate, pH 7.0 at 4°C with a flow rate of 14 ml/h (fraction size 30 minutes). The fractions showing absorbance at 413 nm were pooled and concentrated by ultrafiltration on a UF C-5 membrane (Nucleopore). Polyacrylamide gel electrophoresis of this pool in presence of SDS showed a single band with a molecular weight of about $10,000$.

2.1.2.2. Cleavage by Subtilisin

Sulfite oxidase ($20\text{ }\mu\text{M}$) in 50 mM potassium phosphate, pH 7.8 was incubated with proteinase from *Bacillus subtilis* (Fluka) ($1/25$; w/w) at 37°C . Aliquots were withdrawn and the activity was measured immediately in the cytochrome c and the ferricyanide assay. The digestion was stopped by addition of 0.5 mM PMSF, diluting the sample with the same volume of sample buffer for polyacrylamide gel electrophoresis (180 mM Tris-HCl, 1% SDS, 10% β -mercaptoethanol, 20% Glycerin, pH 6.8) and boiling in a waterbath for 10 minutes. Ten μl samples were analysed by 15% polyacrylamide gel electrophoresis in presence of SDS.

2.1.3. Binding of Cytochrome c to Sulfite Oxidase

The binding affinity of horse cytochrome c (Sigma, type III) for chicken sulfite oxidase was determined by the Hummel and Dreyer (1962) gel filtration technique, as modified by Ferguson-Miller et al. (1976). A column (0.9 x 35 cm) of Bio Gel P-30, fine (Bio Rad) was equilibrated at 4°C with 10 mM cacodylate (Fluka, purum), 20 mM taurine (Fluka, puriss.), pH 6.0, containing various concentrations of cytochrome c (2-16 μ M). A mixture of 4 nmol sulfite oxidase and 20 nmol cytochrome c in a volume of 130 μ l was applied to the column and was allowed to filter through the gel. Non-bound cytochrome c was completely separated from the sulfite oxidase-cytochrome c complex during the run.

The amount of cytochrome c bound to sulfite oxidase was determined from the dithionite reduced spectrum of the sulfite oxidase peak, taking the extinctions coefficients of sulfite oxidase at 555 nm and 550 nm to be 28 and 15.5 $\text{mM}^{-1}\text{cm}^{-1}$, respectively, and those of cytochrome c at 550 nm and 555 nm to be 29.5 and 9.5 $\text{mM}^{-1}\text{cm}^{-1}$, respectively, as determined from the absorption of known mixtures of sulfite oxidase and cytochrome c.

2.1.4. Immunological Methods

2.1.4.1. Production of Antisera

Two white rabbits were immunized with purified sulfite oxidase in the following way: The first rabbit, producing antiserum A, was injected subcutaneously with 0.2 mg of native and 0.2 mg of denatured (boiling for 3 minutes in 1% SDS) sulfite oxidase emulsified with 1 ml of Freund's complete adjuvant (Difco Laboratories). Four weeks later 0.2 mg of native and 0.2 mg of denatured enzyme emulsified with 1 ml of incomplete Freund's adjuvant (Difco Laboratories) was injected subcutaneously. The serum was obtained from the blood of the immunized animal 10 days later. Injection of the antigen in incomplete Freund's adjuvant and bleeding was repeated every 4 weeks.

The second rabbit, producing antiserum B, was treated in the same way, except that only 0.05 mg of native sulfite oxidase was emulsified for every injection.

The sera were separated from clotted erythrocytes by standing at 37°C for several hours, with a small wooden rod in every tube. After cooling at 4°C for 2 hours the rod to which most of the erythrocytes stuck was removed. The serum was centrifuged and the supernatant stored in aliquots at -20°C.

2.1.4.2. Ouchterlony Double Diffusion

The undiluted antisera were tested in the double diffusion test of Ouchterlony (1967). The test was performed in 1,5% agarose (Pharmacia, IEF grade) in phosphate buffered saline (PBS: KH_2PO_4 , 0.2 g/l, $\text{Na}_2\text{HPO}_4 \times 2 \text{ H}_2\text{O}$, 1.15 g/l, NaCl, 8 g/l, KCl, 0.2 g/l). The antigen was diluted with PBS. Diffusion was allowed to proceed for 18 hours at 37°C in a humidity chamber. After formation of the precipitation lines the plates were washed with PBS to remove uncomplexed proteins, dried with a hair-drier and stained with Coomassie brilliant blue.

2.2. CHEMICAL MODIFICATION OF SULFITE OXIDASE

2.2.1. Modification of Carboxyl Groups

2.2.1.1. Reaction with Woodward's Reagent K

Modification of sulfite oxidase with Woodward's reagent K (Fluka, purum) was performed in 10 mM Tris-HCl, pH 7.0 at 20°C. The pH of the reaction mixture was monitored with the help of a pH-stat assembly (Radiometer, Copenhagen) and corrected by the addition of 0.1 M NaOH, as appropriate. Twenty μ M sulfite oxidase, with or without 50 μ M cytochrome c, was incubated with 10 mM Woodward's reagent K. In the control experiment the reagent was omitted. Aliquots of 25 μ l were withdrawn and the reaction was stopped by addition of 50 μ l of 50 mM sodium acetate, pH 7.0. Activity was measured in the cytochrome c and the ferricyanide assay.

After 2 minutes reactions were stopped by addition of 2 volumes of 50 mM sodium acetate, pH 7.0. The samples were applied on a column (0.9 x 25 cm) of Bio-Gel P-100, fine (Bio Rad) and eluted with 50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5. The kinetic constants of the modified sulfite oxidase were measured in the elution buffer. Measurements were performed on an instrument constructed by Bruno Michel (1987) with the assay parameters as specified in table 1 (section 2.2.1.3.).

2.2.1.2. Synthesis of N-Cyclohexyl-N'-(2-(N-[³H]-methylmorpholino)ethyl) carbodiimide p-toluene sulfonate

N-Cyclohexyl-N'-(2-(morpholino)ethyl)carbodiimide was prepared by reacting N-cyclohexyl-N'-(2-(morpholino)ethyl)thiourea (Aldrich) with mercuric oxide (Fluka) according to the method of Sheehan & Hlavka (1956). Before further use, the carbodiimide was distilled under reduced pressure.

[³H]-CMC was synthesized according to the procedure of Banks et al. (1969) and Ariki & Fukui (1978). A sealed tube containing 8.3 nmol of [³H]-methyl iodide (25 mCi, Amersham) was connected to one side of a vacuum line. A test tube containing 0.125 ml (2 mmol, 0.284 g) of unlabeled methyl iodide (Fluka) in liquid nitrogen was connected to the other side. After evacuation of the line, the seal was broken. The labeled methyl iodide was allowed to distill to the unlabeled compound for 30 minutes. Then, the vacuum line was filled with nitrogen and 0.6 ml (2.5 mmol, 0.585 g) of N-cyclohexyl-N'-(2-(morpholino)ethyl)carbodiimide was added to the test tube.

The remaining steps were performed as described by Banks et al. (1969) with the following exceptions: the solid N-cyclohexyl-N'-(2-(N-[³H]-methyl-morpholino)ethyl)carbodiimide iodide was washed with petroleumbenzine 40°-60° (Fluka, p.a.) instead of petroleum ether, commercial silver tosylate (Aldrich) was used and the [³H]-CMC was recrystallized four times from tetrahydrofurane (Fluka, puriss., getrocknet) to which 0.1% of H₂O was added.

The ultraviolet spectrum and the color reaction with dimethyl barbituric acid (Wilchek et al., 1981), of [³H]-CMC were identical to those of unlabeled, commercial CMC (Fluka). The specific activity was measured by the external standard procedure on a Betamatic II (Kontron) instrument in 7 ml of Kontrogel (Kontron). The quench curve used for the dpm-procedure was measured with ³H-standards (Lumac), quenched with up to 7 % of acetone in 7 ml of Kontrogel. The specific activity of [³H]-CMC was 21.2 Ci/mol.

2.2.1.3. Reaction of Sulfite Oxidase with Carbodiimides

Modification of sulfite oxidase with CMC and EDC was performed at 20°C if nothing else is specified. Buffer composition and concentration of protein and reagent are given in the figure legends. The reagents were of the best quality available from Fluka. CMC and EDC were dissolved

in a minimum volume of H_2O immediately before addition to the protein solution. To follow the inhibition of the sulfite oxidase activity, samples were withdrawn, diluted 1 : 500 with 0.1 M Tris-HCl, pH 7.5, and assayed immediately.

The number of molecules of CMC incorporated per molecule of sulfite oxidase was determined with the help of $[^3\text{H}]$ -CMC. Sulfite oxidase (9 μM) was incubated with various concentrations of $[^3\text{H}]$ -CMC (1.7, 4.5, 10, 24 mM) in 10 mM potassium phosphate, pH 7.0, at 30°C. At different time points (usually after 30, 60, 90 and 120 minutes) small aliquots were withdrawn and assayed for cytochrome c reductase activity. A larger sample (0.25 ml) of the reaction mixture was withdrawn and the reaction was stopped by the addition of 0.25 ml of 0.2 M sodium acetate, pH 6.0, and freezing of the sample in a deep freezer (-70°C).

For determination of the incorporated radioactivity samples were thawed and applied immediately onto a Bio-Gel P-6, fine, column (1 x 36 cm) and eluted (7 ml/h) with 0.1 M potassium phosphate, pH 6.0. After about two hours the protein eluted from the column. Unbound radioactivity was completely separated by this step. The protein peak was pooled. The concentration of sulfite oxidase in this pool was calculated from the spectrum, the amount of radioactivity incorporated was determined on 0.2 ml aliquots of the pool.

For determination of the kinetic constants of modified sulfite oxidase, the enzyme was incubated with CMC in 10 mM potassium phosphate, pH 7.0, at 30°C or with EDC in 0.1 M potassium phosphate, pH 6.0. The reaction was stopped by bringing the samples to 50 mM Tris-HCl, pH 7.5, by the microcentrifuge desalting method described by Helmerhorst & Stockes (1980). The kinetic constants were determined by the method developed by Michel (1987). See table 1 for details.

Table 1: Assay parameters for measurement of kinetic constants
Instrumentation and measuring protocol as specified by Michel (1987).

Time	[S]min	2	to	4 sec
Time	[S]max	2	to	10 sec
Interval	.5 /	Integration		.5
Max.Std.dev.	3 /	Nonlinearity		15%
Range 1	from	548	to	552 nm
Range 2	from	416	to	420 nm
Int. Ref.	from	580	to	586 nm
[SOX]				6.6 nM
in assay :				.5 nM
[Cytochrome c]				61.2 μ M
Max.conc. in assay				10 μ M
No. of points:	20	Replicates : 3		
Delta epsilon 1 :		16.2	1/mM cm	
Delta epsilon 2 :		43.1	1/mM cm	
Assay volume :		0.4	ml	

To measure the distribution of incorporated CMC on the domains of sulfite oxidase, the enzyme (20 μ M) was modified in 10 mM potassium phosphate, pH 7.0, at 30°C with different concentrations (1-10 mM) of the radioactive reagent. Aliquots of the reaction mixture were withdrawn and assayed for cytochrome c reductase activity. The reaction was stopped (after 20-75 minutes) by the microcentrifuge desalting method, bringing the samples to 50 mM potassium phosphate, pH 7.8. Digestion with chymotrypsin (1/15, w/w) was performed at 37°C. Aliquots were withdrawn, brought to 1 mM PMSF, diluted with the same volume of sample buffer for polyacrylamide gel electrophoresis (see section 2.1.2.2. for details), boiled in a waterbath for 10 minutes, and stored at -70°C.

Twenty μl of these samples were run on a 15% polyacrylamide electrophoresis gel in presence of SDS. The Coomassie blue stainable bands corresponding to sulfite oxidase and the heme core of the enzyme (according to Guiard & Lederer, 1977) were cut out and incubated with 11.2 ml of a mixture of Lipoluma (Lumac) / Lumasolve (Lumac) / H_2O , 10/1/0.2. The samples were allowed to stand at room temperature in the dark for several days and were then counted. The background was determined by gel pieces cut out next to every protein band.

2.2.2. Modification of Histidine Residues

Modification was achieved with DEP (Fluka purum) at 20°C under the conditions specified in the figure legends. DEP was dissolved in dry ethanol immediately before use. The concentration of DEP in ethanolic solution was determined photometrically according to Miles (1977). To measure the extent of inhibition, samples were withdrawn, diluted 1:2000 with 0.1 M Tris-HCl, pH 7.5, and assayed immediately. The number of histidine residues modified was obtained from the difference absorption of modified minus native enzyme, $\Delta\epsilon_{240} = 3.2 \text{ mM}^{-1}\text{cm}^{-1}$, according to Miles (1977).

3. RESULTS

3.1. ISOLATION AND STORAGE OF CHICKEN LIVER SULFITE OXIDASE

The results of a typical isolation procedure are summarized in table 2. The starting material was 400 g of an acetone powder obtained from 2 kg of chicken liver. The amount of sulfite oxidase extracted from the chicken liver was much smaller than reported by Kessler and Rajagopalan (1972), but the yield of the purification steps and the purity of the final preparation were slightly higher.

Table 2: Purification of sulfite oxidase from chicken liver

Procedure	Total units	Total protein (mg)	Specific activity (U/mg)	Yield (%)	A_{413}/A_{280}
Acetone powder	673,050	91,186	7.4	100	
Heat step *	413,000	40,658	10.2	61	
Ammonium sulfate precipitate	565,150	27,860	20.3	84	
Acetone precipitate	472,600	3,019	156	70	
DE-52 ion exchange	245,520	119.5	2,054	36	
Affinity column					
Pool A	88,652	10.2	8,691	13	0.81
Pool B	125,970	19.4	6,493	18	0.77

* Activity values for the heat step supernatant reflect some inhibition by sulfate ions carried over into the assay cuvette (Kessler & Rajagopalan, 1974).

Figure 7 depicts the elution profile of the affinity column. Sulfite oxidase bound very tightly to the column, while a great part of the impurities was eluted with the starting buffer. The enzyme started to elute when the gradient reached a concentration of about 10 mM phosphate. The affinity purification has two advantages compared to the gel filtration employed by Johnson & Rajagopalan (1976) : handling of the affinity column at 4°C is less delicate than handling of a gel filtration column, and affinity chromatography is much faster.

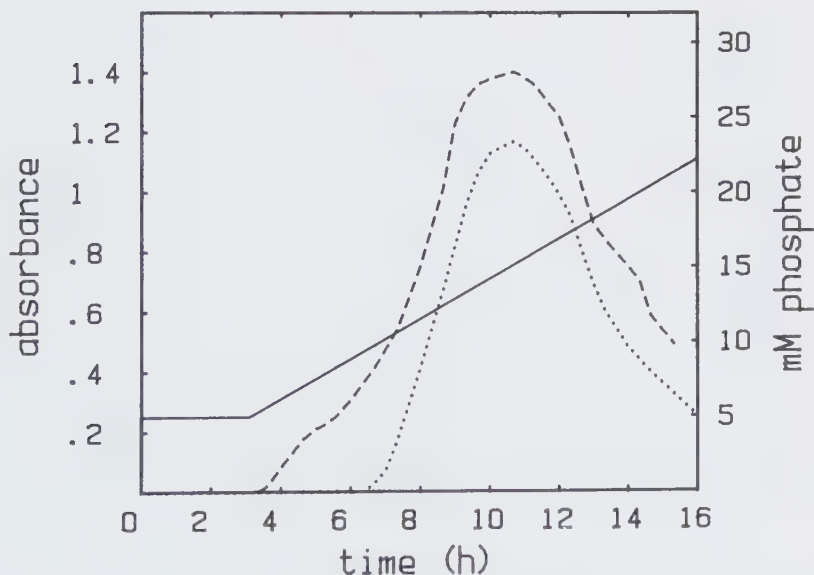


Figure 7: Affinity chromatography of sulfite oxidase on cytochrome c Sepharose

(.....) absorbance at 413 nm;

(-----) absorbance at 280 nm;

(—————) phosphate concentration;

see section 2.1.1.1. for details.

The enzyme was stored in the elution buffer from the affinity column at -70°C for several weeks without appreciable loss of activity. In my experience, storage in liquid nitrogen in 50% (v/v) ethylene glycol according to Speck et al. (1981) occasionally led to complete loss of activity within weeks, probably because of oxidizing impurities present in commercial ethylene glycol as described by Washabaugh & Collins (1983).

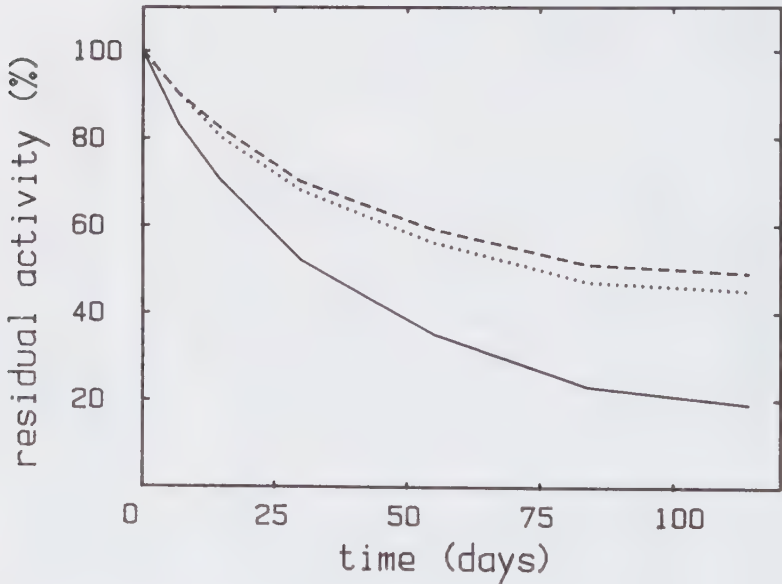


Figure 8: Inactivation of sulfite oxidase by storage at 4°C
(—) in 50 mM potassium phosphate, pH 7.8
(- - -) in 50 mM potassium phosphate, 0.2 mM PMSF, pH 7.8
(.....) in 50 mM potassium phosphate, pH 7.8, containing 2 μg each of antipain, chymostatin, leupeptin, and pepstatin per mg of sulfite oxidase. The activity was measured in the cytochrome c assay.

Storage of sulfite oxidase at 4°C caused inactivation of the enzyme within weeks. The influence of the addition of 0.2 mM PMSF and of a mixture of antipain, chymostatin, leupeptin and pepstatin (1 mg/ml each) in 20% dimethylsulfoxid (2 µl/mg of sulfite oxidase) is shown in figure 8. Best results were obtained in presence of PMSF. The aliquots thawed from -70°C were therefore brought to 0.2 mM PMSF and used within two weeks.

3.2. CLEAVAGE OF SULFITE OXIDASE BY SUBTILISIN

Cleavage of chicken liver sulfite oxidase by proteinase from *Bac. subtilis* led to a pattern of inactivation similar to that reported for cleavage of the rat enzyme by trypsin (Southerland et al., 1978). Figure 9 shows a fast decrease of the cytochrome c reductase activity, while the ferricyanide reductase activity is lost much slower. This result indicates cleavage of the enzyme into the molybdenum-domain with a molecular weight of about 45,000, which is responsible for the ferricyanide reductase activity, and the heme-domain with a molecular weight of about 10,000.

This digestion pattern was confirmed by polyacrylamide gel electrophoresis in presence of SDS as presented in figure 10. The enzyme seems to be cleaved into two different molybdenum- and heme-domains. The appearance of two different populations of the heme- and the molybdenum-domain indicates that there are two different cleavage sites for subtilisin in the hinge peptide connecting the two domains.

This finding contrasts with that reported by Guiard & Lederer (1977). They found that chicken liver sulfite oxidase was degraded by subtilisin to a heterogeneous mixture of small fragments. The source of the proteinase and its subtype were not described.

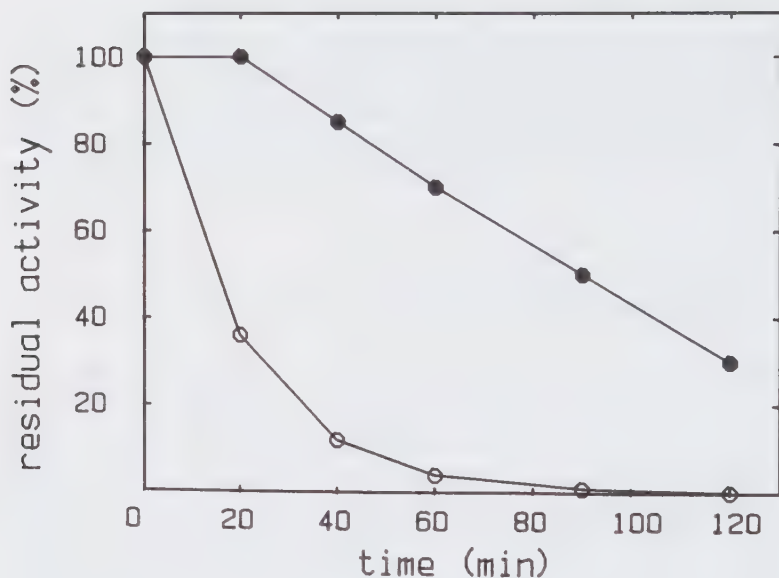


Figure 9: Digestion of sulfite oxidase by subtilisin

At the time points indicated aliquots were withdrawn and assayed by the cytochrome c assay (O) and by the ferricyanide assay (●). See section 2.1.2.2. for details.

It is surprising that subtilisin, which is a rather unspecific protease leads to such a specific cleavage. The reason may be the great mobility and accessibility of the hinge region for the protease, compared to the rest of the protein.

Inactivation of chicken sulfite oxidase by trypsin followed the pattern as described by Guiard & Lederer (1977). Elastase, thermolysin, V8 protease, clostripain and papain inhibited the electron transfer to cytochrome c and to ferricyanide to the same extent and led to a mixture of small peptides without even a transient appearance of the intact domains.

The different behavior of chicken and rat sulfite oxidase towards trypsin, chymotrypsin and papain (Guiard & Lederer, 1977; Southerland et al., 1978) may be due to sequence differences in the carboxy-terminal part of the two heme-domains (Guiard & Lederer, 1979; Rajagopalan, 1980).

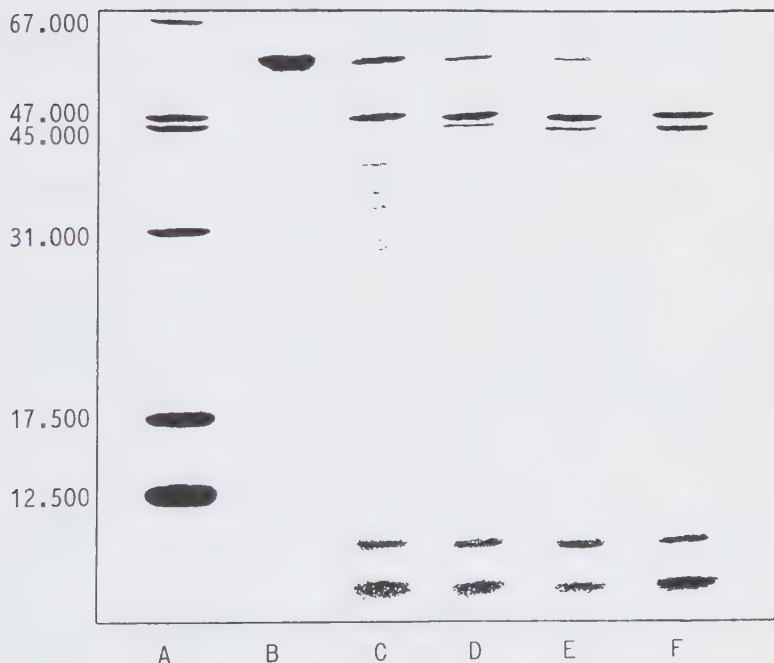


Figure 10: Polyacrylamide gel electrophoresis in presence of SDS of chicken sulfite oxidase digested by subtilisin

Lane A: molecular weight marker; lane B: native sulfite oxidase; lane C, D, E, F: aliquots of the digestion mixture withdrawn after 20, 40, 60, and 90 minutes, respectively. See section 2.1.2.2. for details.

3.3. BINDING OF CYTOCHROME c TO SULFITE OXIDASE

The result of the binding measurement is shown in figure 11. The negative reciprocal of the slope of the curve is the dissociation constant (K_d) for the binding of horse cytochrome c to chicken sulfite oxidase. K_d was $7.5 \mu\text{M}$ at an ionic strength of 10 mM . The line extrapolates to an r value of 1.94 , indicating two molecules of cytochrome c bound per heme b, or four molecules per sulfite oxidase dimer. Furthermore, it appears that the two binding sites were equal and non-interacting. Additional weak binding sites cannot be excluded for lack of experimental data at higher r values (figure 11).

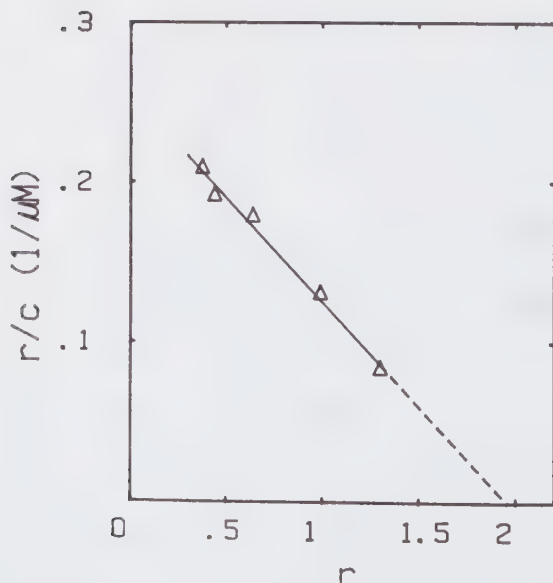


Figure 11: Binding of horse cytochrome c to chicken sulfite oxidase
Data are plotted according to the equation

$$r / c = 1 / K_d (r - n)$$

where r is the ratio of cytochrome c bound to sulfite oxidase, c is the concentration of free cytochrome c in the equilibration buffer, K_d is the dissociation constant and n is the number of binding sites.

3.4. RABBIT-ANTI-CHICKEN SULFITE OXIDASE ANTISERA

Two antisera against chicken sulfite oxidase raised in rabbits were found to inhibit the cytochrome c reductase activity, but not the ferricyanide reductase activity (figure 12). This may indicate that the epitopes recognized by the two antisera are on the heme-domain. Indeed, sulfite oxidase heme-domain could displace the antibodies from the enzyme. Cytochrome c reductase activity was partially regained in the presence of free heme-domain, as shown in Table 3.

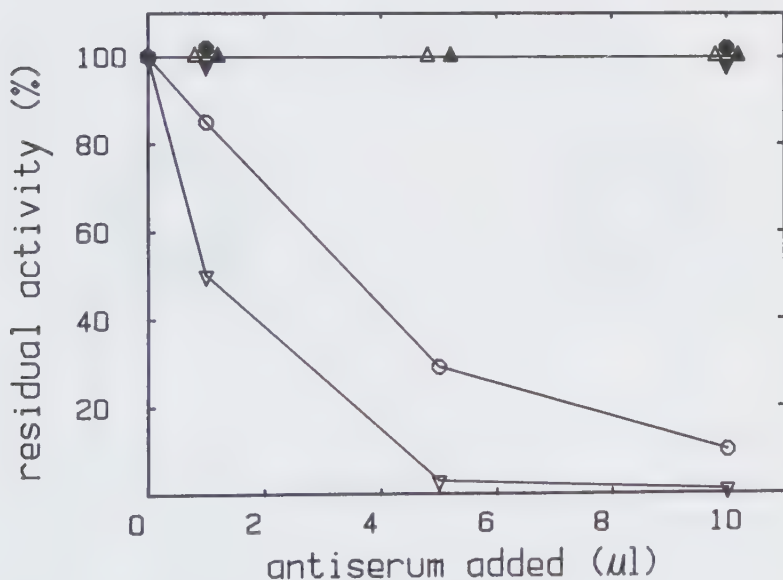


Figure 12: Inhibition of sulfite oxidase by antiserum

The indicated volumes of the serum were added to the enzyme assays (see 2.1.1.2.). The mixture was incubated for 10 minutes at room temperature before starting the reaction by addition of the electron acceptor. Activity was measured by the cytochrome c assay (open symbols) and by the ferricyanide assay (closed symbols) in the presence of antiserum A (▽ ▼), antiserum B (○, ●), and rabbit normal serum (Δ, ▲).

Antibodies raised against rat sulfite oxidase molybdenum-domain did inhibit both, the cytochrome c reductase and the ferricyanide reductase activity (Kuwahara et al., 1982). Thus, in principle, it is possible to inhibit the reaction with ferricyanide, although ferricyanide is much smaller than cytochrome c.

Table 3: The influence of isolated heme-domain on the inhibition of sulfite oxidase by antiserum

Antiserum was added to the cytochrome c assay containing the indicated concentrations of sulfite oxidase and heme-domain. After incubating for 10 minutes at room temperature the reaction was started by addition of sodium sulfite.

sulfite oxidase (nM)	heme-domain (nM)	residual activity (%) with		
		antiserum A 1/2000	B 1/600	normal serum 1/2000
5	0	59	53	100
5	5	68	67	100
5	10	72	74	100
5	20	77	77	100
0	10	0	0	0

The Ouchterlony double diffusion test (figure 13) showed a precipitation line with the intact enzyme, but not with the heme-domain. It seems that the antibodies were directed against a single epitope on the heme-domain, hence crosslinking and precipitation was observed only with the native sulfite oxidase dimer, containing two non-covalently linked heme-domains.

All these results support a single epitope on the heme-domain recognized by an antibody present in the antisera.

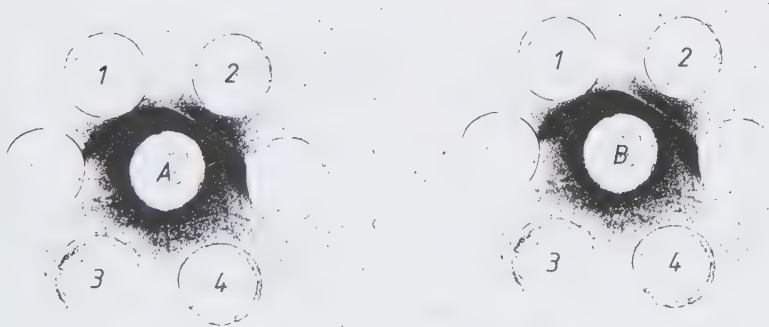


Figure 13: Ouchterlony double diffusion test with antiserum A and B
Well A contained undiluted antiserum A, well B antiserum B;
well 1: sulfite oxidase, 0.1 mg/ml; well 2: sulfite oxidase,
0.03 mg/ml; well 3: heme-domain, 0.1 mg/ml; well 4: heme-
domain, 0.03 mg/ml.

3. 5. MODIFICATION OF CARBOXYL GROUPS WITH WOODWARD'S REAGENT K

Woodward's reagent K was the most powerful agent to destroy the enzymatic activity. The result of the inactivation study is shown in figure 14. The activities with both substrates were impaired in presence or absence of cytochrome c in the incubation mixture. It was not possible to inhibit selectively the cytochrome c reductase activity. The kinetic constants of the modified enzyme indicate that binding of cytochrome c is not affected by the modification (Table 4).

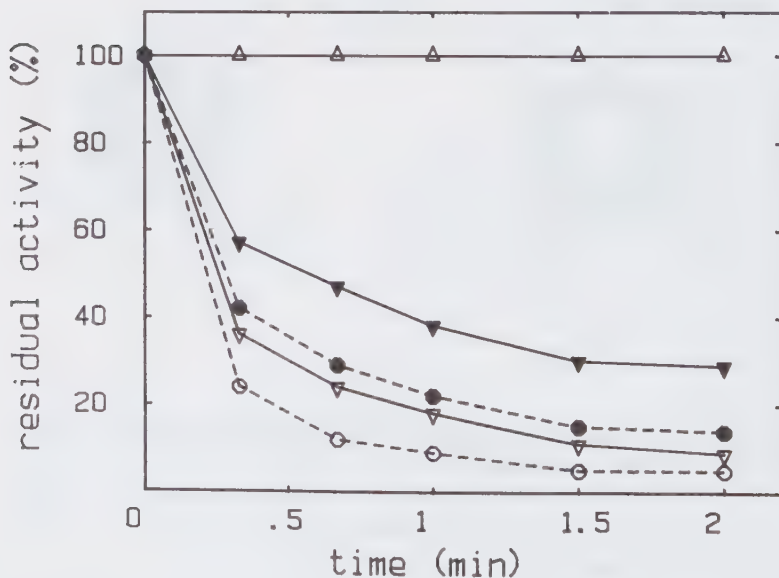


Figure 14: Inactivation of sulfite oxidase with Woodward's reagent K
Sulfite oxidase (20 μ M) was treated with 10 mM Woodward's reagent K in the presence (—) or absence (----) of 50 μ M cytochrome c . Residual activity was measured in the cytochrome c assay (open symbols), in the ferricyanide assay (closed symbols), and in a control experiment without reagent (Δ).

Table 4: Kinetic constants of sulfite oxidase modified with Woodward's reagent K

Samples were prepared and measured as described in section 2.2.1.1.

sulfite oxidase modified	K_m (μM)	V_{max} (s^{-1})
in presence of cytochrome c	0.75	6.1
in absence of cytochrome c	0.70	9.1
control without reagent	0.78	81.5

Because it was not possible to inhibit selectively the reaction with cytochrome c, this reagents was not suitable to probe carboxyl groups in the reaction with cytochrome c.

3.6. MODIFICATION OF CARBOXYL GROUPS BY CARBODIIMIDES

3.6.1. Selectivity of the Reagent

Carbodiimides react with a number of organic functional groups. In aqueous solution at acidic pH values the predominant side chains of proteins reacting with carbodiimides are carboxyls, sulfhydryls, and phenolic hydroxyls (Carraway & Koshland, 1968; Carraway & Triplett, 1970). Since carbodiimides react with the protonated carboxyl group (Hoare & Koshland, 1967) the pH-dependence of the loss of enzyme activity may reveal the nature of those side chains modification of which leads to inactivation.

Figure 15 shows that the rate of inhibition of the enzymatic activity increases at lower pH. This is in agreement with the fact that carbodiimides react preferentially with protonated carboxyl groups and that their modification inactivates the enzyme. No pH-dependence in the slightly acidic pH region is expected for the attack at cysteine and tyrosine residues, the pK_a 's of which are in the alkaline region.

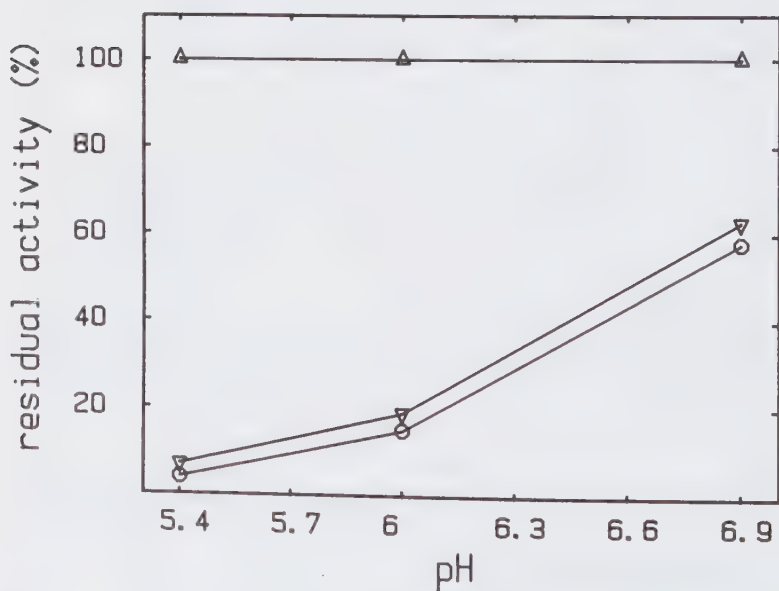


Figure 15: pH-Dependence of the inactivation of sulfite oxidase by carbodiimides

Sulfite oxidase (20 μ M) was treated with 50 mM EDC (▽), with 50 mM CMC (○), or without carbodiimide (Δ) for 20 minutes in 10 mM cacodylate-HCl at the indicated pH-values. Activity was measured by the cytochrome c assay.

The contribution of tyrosine modification to loss of activity was also estimated by treating the modified enzyme with hydroxylamine to regenerate free tyrosine (Carraway & Koshland, 1968). Incubation of modified sulfite oxidase with hydroxylamine did not reactivate the enzyme (figure 16). If tyrosine residues are modified at all, their modification is not responsible for inactivation.

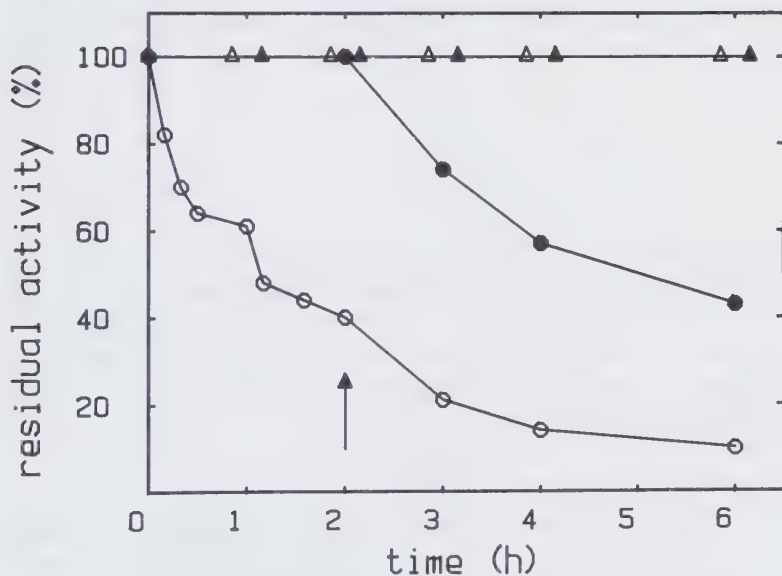


Figure 16: Reactivation of modified sulfite oxidase with hydroxylamine
20 μ M sulfite oxidase in 250 μ l of 0.1 M potassium phosphate, pH 6.2 was treated with two portions of 20 μ l each of 130 mM CMC (added at the beginning and after 1 hour), and the activity of samples taken at the time points indicated was measured. Modification was allowed to proceed at room temperature. After 2 hours (arrow), the reaction mixture was cooled to 4°C and hydroxylamine was added to a final concentration of 0.1 M. Samples were withdrawn, separated from the reagent by gel filtration and assayed for cytochrome c reductase activity (open symbols) and ferricyanide reductase activity (closed symbols). In a control CMC was omitted (Δ).

To exclude a contribution of sulfhydryl group modification to the inactivation of sulfite oxidase, the enzyme was incubated with 5,5'-dithio-bis-(2-nitrobenzoic acid), a reagent specific for sulfhydryl groups. No inactivation was observed with 3 mM reagent (data not shown).

Carbodiimides may occasionally cleave peptide bonds (Paterson & Leach, 1980). Under the experimental conditions used, sulfite oxidase remained intact as revealed by analyzing the enzyme by polyacrylamide gel electrophoresis in presence of SDS before and after treatment with CMC (not shown).

3.6.2. Effect of Cytochrome c

Addition of substrates to enzyme modification reactions often prevents or slows down inactivation. In the case of cytochrome c peroxidase the rate of inactivation by EDC is dramatically decreased if cytochrome c is present in the modification reaction (Waldmeyer et al., 1982). This observation indicates that the amino acid side chains modified during inactivation are at, or near to, the binding site of the substrate.

Modification of sulfite oxidase by CMC in presence and absence of cytochrome c is depicted in figure 17. During modification the enzyme was, on average, 90% saturated with cytochrome c. The rate of modification was virtually the same for the sulfite oxidase-cytochrome c complex and for the free enzyme. The small difference at 5 mM CMC might be due to the higher concentration of modifiable carboxyl groups in the presence of cytochrome c. The site of inactivation by CMC seems equally accessible in the complex and in the free enzyme.

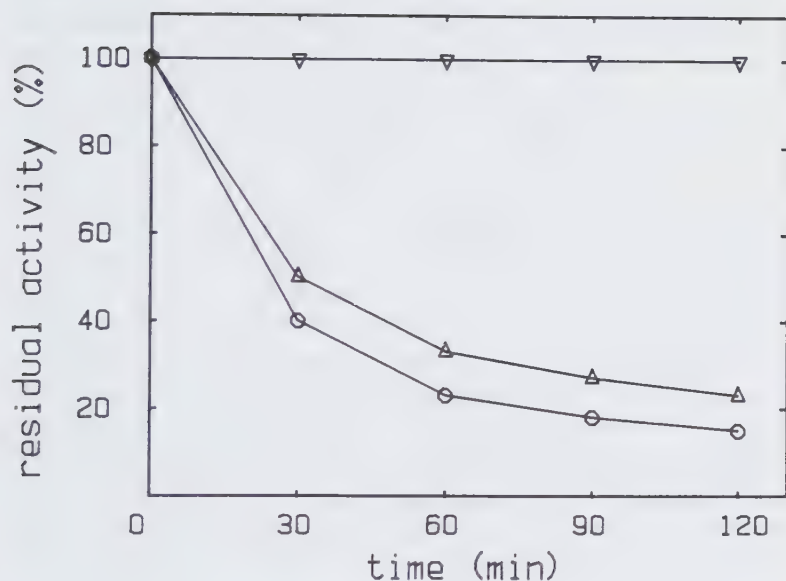


Figure 17: Inactivation of sulfite oxidase by CMC in presence and absence of cytochrome c
20 μ M sulfite oxidase with (Δ) or without ($\circ$) 100 μ M cytochrome c present was incubated at 30°C with 5 mM CMC in 10 mM potassium phosphate, pH 6.0. Aliquots were withdrawn at the time points indicated and assayed immediately by the cytochrome c assay (open symbols) and by the ferricyanide assay (closed symbols).

3.6.3. Effect of Nucleophiles

To determine the number of carboxyl groups modified by a carbodiimide the O-acylisourea intermediate may be reacted with a nucleophile (see scheme 1 in section 4.3). Diaminoethane, glycine, and taurine were used frequently. The number of modified residues can be estimated by amino acid analysis of the product, or if a radioactive nucleophile is used, by determining of the radioactivity incorporated.

In order to estimate the number of carboxyl groups modified in sulfite oxidase, the reaction with EDC was performed in the presence of [^3H]taurine. The reaction of the O-acylisourea with taurine yields the stable amide $-\text{CO}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{SO}_3^-$. Surprisingly, only 1.2 mol of [^3H]taurine per mol of sulfite oxidase was incorporated after 2 hours when the nucleophile was present in excess and EDC was added in portions at timed intervals, as shown in figure 18. The figure also demonstrates that the cytochrome c reductase activity of the enzyme decreased at about the same rate with or without taurine present during modification with DEP. The same result was obtained when semicarbazide or diaminoethane were used as nucleophiles (data not shown).

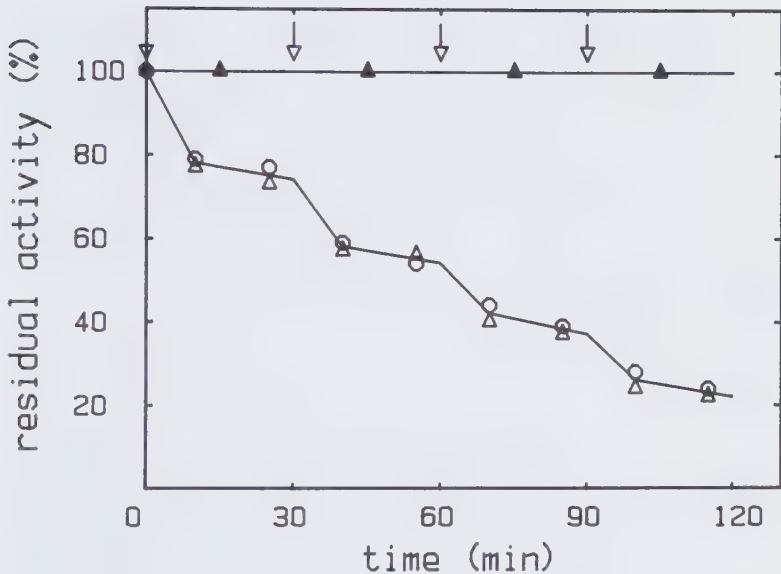


Figure 18: Inactivation of sulfite oxidase by EDC in the presence and absence of taurine

20 μ M sulfite oxidase in 37 μ l of 0.1 M potassium phosphate, pH 6.0, was treated with 4 x 2 μ l of 95 mM EDC added at the time points indicated by arrows. Modification was performed in the absence (Δ) or presence ($\circ$) of 0.1 M [3 H]taurine (8 μ Ci). Activity was measured by the cytochrome c assay (open symbols) or by the ferricyanide assay (closed symbols). After 2 hours reaction time the product was freed from excess reagents by gel filtration and the amount of covalently bound taurine was determined from the incorporated radioactivity.

3.6.4. Kinetics of Inactivation of Sulfite Oxidase by CMC

The time course of inactivation of sulfite oxidase by CMC was followed in 10 mM potassium phosphate, pH 7.0. Despite of the great molar excess of the reagent over the enzyme, a semilogarithmic plot of residual activity versus time did not yield a straight line. This is apparently due to the instability of the reagent in the buffer used. Its half-life is dependent on the buffer system, the concentration of the buffer, the pH and the temperature. In 10 mM potassium phosphate, pH 7.0 the half-life found by Metz and Brown (1969) for CMC was 4.4 hours, corresponding to a first-order rate constant of $k' = 2.63 \times 10^{-3} \text{ min}^{-1}$.

The fraction (A/A_0) of enzyme activity remaining at time t may be expressed as

$$\ln (A/A_0) = - (k/k') I_0 (1 - e^{-k't})$$

where I_0 is the initial concentration of the reagent, k is the bimolecular rate constant for the reaction of the enzyme with the reagent, and k' is the pseudo first-order rate constant of hydrolysis of the reagent (Topham, 1985). The above equation is valid only under the assumption that the modified enzyme is completely inactive and that the decrease in concentration of reagent due to reaction with the enzyme can be neglected.

If the data obtained for inactivation with CMC were plotted versus $t' = (1 - e^{-k't}) / k'$, straight lines were obtained, as shown in figure 19 A. According to this diagram, the rate of inactivation of sulfite oxidase depends linearly on the concentration of CMC.

At low concentrations of the reagent, there is a linear relationship between the apparent rate constant k_{app} of inactivation (slope of figure 19 A) and the concentration of the reagent, as indicated by figure 19 B. But at higher concentrations of CMC the curve of figure 19 B seems to saturate, suggesting the formation of a reversible enzyme-reagent complex prior to irreversible modification and inactivation of the enzyme.

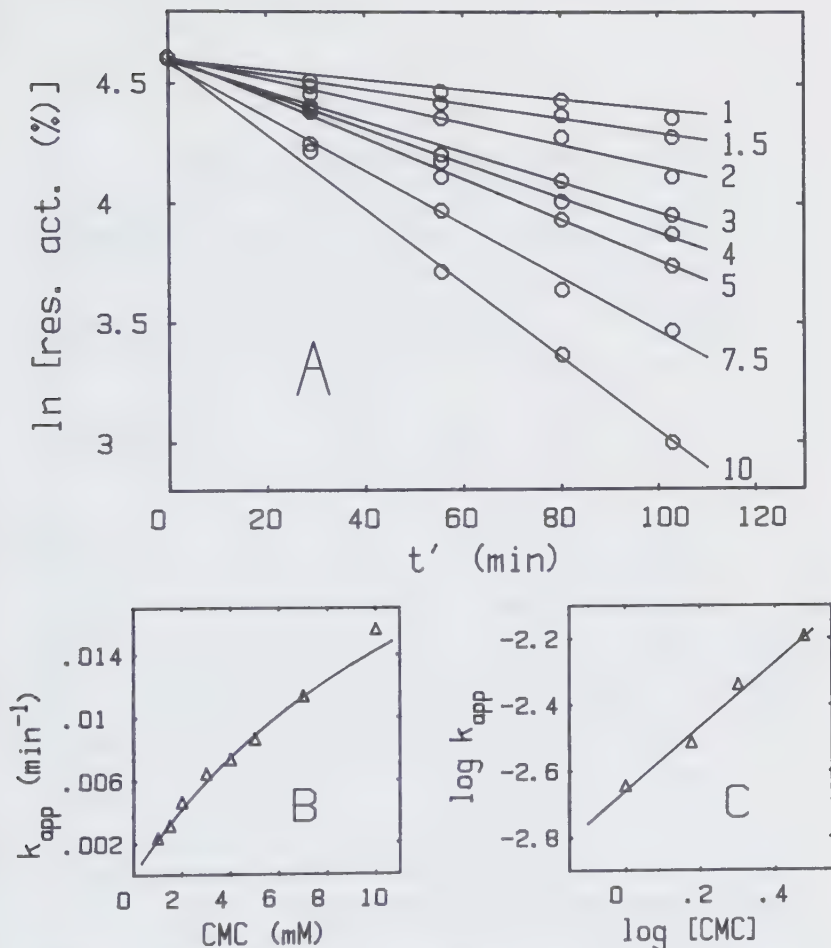


Figure 19: Inactivation of sulfite oxidase by CMC

The enzyme ($10 \mu\text{M}$) was incubated with different concentrations of the reagent (given in mM in panel A) in 10 mM potassium phosphate, pH 7.0 at 30°C . Aliquots were withdrawn after 30, 60, 90, and 120 minutes and assayed for cytochrome c reductase activity.

Panel A: Semilogarithmic plot of residual activity versus t' ; t' was calculated with $k' = 2.63 \times 10^{-3} \text{ min}^{-1}$, as described in the text.

Panel B: Linear plot of the apparent pseudo first-order constants k_{app} (obtained from the curves in panel A) versus the corresponding concentrations of the reagent.

Panel C: double-logarithmic plot of the first (linear) part of the curve in panel B.

The modification reaction can be described by the scheme:

$$\text{sulfite oxidase} + \text{CMC} \xrightleftharpoons{K_1} \text{sulfite oxidase-CMC} \xrightarrow{k} \text{sulfite oxidase}^*$$

where K_1 is the dissociation constant of the reversible complex and k is the rate constant of inactivation. From the scheme, the following equations can be derived (Kitz & Wilson, 1962):

$$k_{\text{app}} = (k [\text{CMC}]) / (K_1 + [\text{CMC}]), \text{ or } 1/k_{\text{app}} = 1/k + K_1 / (k [\text{CMC}]).$$

The equation has the form of the Michaelis-Menten equation.

Fitting the data shown in figure 19 B by a non-linear least squares analysis yields k and K_1 of 0.038 min^{-1} and 16.4 mM , respectively.

The reaction-order n with respect to CMC is given by the equation $k_{\text{app}} = k [\text{CMC}]^n$, where k is a proportionality constant. Since the plot of k_{app} against $[\text{CMC}]$ is linear at low reagent concentration (figure 19 B), n must be around one. This was verified by plotting $\log k_{\text{app}}$ against $\log [\text{CMC}]$ which gave a straight line with a slope of 0.98 (figure 19 C). This result suggests that the reaction of one molecule of CMC per sulfite oxidase monomer is involved in the rate limiting step of the inactivation process.

3.6.5. Incorporation of [^3H]-CMC and its Influence on Activity and Kinetic Constants of Sulfite Oxidase

Since very little [^3H]taurine was incorporated into the carbodiimide modified enzyme (see 3.6.3.) we assume that the transient O-acylisourea derivatives are preferentially stabilized by rearrangement to the N-acylurea derivative (scheme 1, section 4.3.). Using [^3H]-CMC the number of N-acylurea derivatives formed could be estimated.

The relationship between loss of enzymatic activity and number of residues modified with [^3H]-CMC is shown in figure 20. About one residue was modified very fast, and without any loss of activity. Thereafter the residual activity dropped in a non-linear fashion with increasing modification indicating that there are groups of residues which react at different rates.

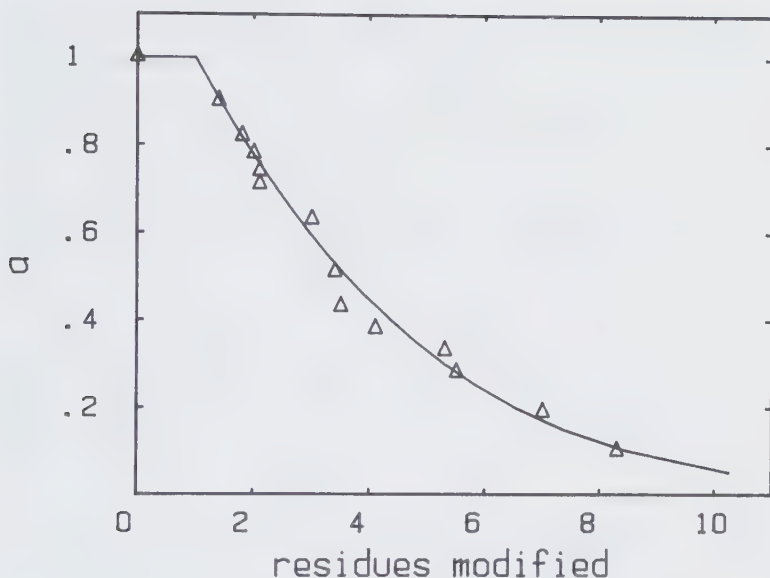


Figure 20: Relationship between residual enzymatic activity and the number of acidic residues modified

The enzyme was modified with [^3H]-CMC as described in section 2.2.1.3. The fraction of residual enzymatic activity a was plotted versus the number of residues modified per sulfite oxidase monomer. The solid line was calculated according to the equation

$$m = n - pa^{1/i} - (n - p) a^{\alpha/i}$$

with $n = 50$, $p = i = 1$, and $\alpha = 0.062$ (see text for details).

The data of figure 20 were analyzed according to Tsou (1962) and Horiike & McCormick (1979). In this method of analysis the number of essential groups is determined from quantitation of the number of groups modified (calculated from the ^3H -radioactivity incorporated) and the residual activity in the samples of partially modified enzyme. We made the simplest possible assumption to explain the non-linearity of figure 20:

the enzyme has n modifiable carboxyl groups among which p carboxyl groups, of which i are essential, react with a pseudo-first-order rate constant k_1 , and $n - p$ residues are not essential and they all react at a pseudo-first-order rate constant αk_1 . Assuming that the modification of any one of the essential residues results in complete loss of activity, the fraction of those molecules remaining that have full activity, denoted by a , will be equal to the fraction of activity remaining. Then the number m of residues modified per molecule is given by the expression (Tsou, 1962):

$$m = n - p a^{1/i} - (n - p) a^{\alpha/i} \quad (1).$$

When the data of figure 20 are fitted to equation (1) by a non-linear least squares analysis, three possible fits with one, two, or three essential residues were obtained. The three fits, A to C shown in table 5 had the same accuracy.

Table 5: Least squares analysis of the data of figure 20 according to equation (1)

Parameter	result of fit		
	A	B	C
i	1	2	3
p	1	2	3
n	50	58	72
α	0.062	0.101	0.118
mean			
root square	0.263	0.258	0.260

Unfortunately it was not possible to determine experimentally the number n of modifiable carboxyl groups. The enzyme became insoluble when more than ten carboxyl groups were modified per sulfite oxidase monomer. The upper limit of n is the sum of glutamic plus aspartic acid residues.

This number cannot be obtained from amino acid analysis because glutamine and asparagine are converted to glutamic acid and aspartic acid during acidic hydrolysis of the protein. Therefore, the sum of glutamic plus aspartic acid residues of the native enzyme was estimated from the isoelectric point and the number of histidine, lysine and arginine.

Sulfite oxidase is an acidic protein (Ono & Ito, 1984). The isoelectric point of the chicken enzyme, estimated from isoelectric focussing is 5 ± 0.2 (not shown). Amino acid analysis yields 12.5 histidine, 16.5 lysine and 32 arginine per monomer (Kessler & Rajagopalan, 1972). At pH 5 the 61 basic residues are fully protonated and equal to the number of COO^- -groups. Assuming an average pK_a of 3.75 for the carboxyl groups (Thomas, 1974) we obtain 64.5 acidic residues. This number may be in error by ± 3 (the error of the published amino acid analysis not included).

It has also to be borne in mind that the present analysis is based on the untested assumption that modification of an essential carboxyl group completely abolishes the enzymatic activity whereas modification of *n* - *i* non-essential carboxyl groups is not inhibitory. This assumption may not hold after several of the non-essential residues are modified; the method has been criticized for this reason (Rakitzis, 1985). In the present case the criticism might be less severe since the number of non-essential residues are modified much more slowly than the essential residue(s).

The first residue which is modified very fast and without loss of activity may be the single residue modified with taurine.

The kinetic constants of modified enzyme preparations with different residual activity in the cytochrome *c* assay are given in table 6. K_m -values are much less altered than V_{max} -values, which indicates that binding of cytochrome *c* to the enzyme is not very much affected. It rather seems that modification of the essential carboxyl group(s) interferes with the intramolecular or the intermolecular electron transfer.

The observation of linear Eadie-Hofstee plots for different modified derivatives indicates that we are dealing with mixtures of fully active and inactive enzyme and not with mixtures of derivatives of different partial activity.

Table 6: Kinetic constants of carbodiimide modified sulfite oxidase

modification conditions	% resid. activ.		
	cytochrome c assay	K_m (μM)	V_{max} (s^{-1})
10 M sulfite oxidase modified			
at 30°C in 10 mM potassium phosphate, pH 7.0			
control	100	0.92	85
1 mM CMC for 10 minutes	94	0.92	80
5 mM CMC for 90 minutes	48	1.36	40
30 mM CMC for 75 minutes	10	1.78	7
20 μM sulfite oxidase modified			
at 20°C in 100 mM potassium phosphate, pH 6.0			
control	100	0.90	81
4 x 10 mM EDC, 30 minutes each	30	0.93	26

The distribution of the 3H -radioactivity incorporated at different degrees of inactivation is shown in table 7. Three samples of the enzyme were modified to the extend indicated in table 7. The samples were digested with chymotrypsin for different periods of time and the digests analysed by polyacrylamide gel electrophoresis in presence of SDS. Bands containing the intact enzyme and the heme-domain were counted for 3H -radioactivity. The molybdenum-domain is cleaved into small fragments by chymotrypsin and its radioactivity content could not be determined.

Table 7: Incorporation of ^3H -CMC to the domains of sulfite oxidase

residual activity (%)	residues incorp. per SOX	digest. by chymotr. (h)	radioactivity sulfite oxidase		radioactivity heme-domain	
			cpm	%	cpm	%
100	0.3	0	172	100	0	0
		3	80	46	78	45
		6	58	34	85	49
		9	44	25	108	63
61	3	0	1439	100	0	0
		3	287	20	247	17
		6	164	11	323	22
		9	95	7	370	26
30	5.5	0	3226	100	0	0
		3	1062	33	443	14
		6	623	19	495	15
		9	303	9	615	19

When about 0.3 residues are modified more than 60% of the radioactivity initially present is found in the heme-domain after 9 hours of digestion. This indicates that the first residue, the modification of which is very fast and does not decrease the enzymatic activity, is located on the heme-domain.

When the residual activity falls to 61% and three residues are modified, the radioactivity in the heme-domain drops to 26%. If one assumes that the fast reacting residue is on the heme-domain and the non-essential residues are statistically distributed according to the size of the two domains, the radioactivity in the heme-domain is expected to be 56% if the essential residue is on the heme-domain and 43% if it is on the molybdenum-domain. The result shown in table 7, suggests that the essential residue is on the molybdenum-domain. The amount of radioactivity in

the heme-domain is smaller than expected. The reason might be a lower number of non-essential modifiable groups on the heme-domain and partial digestion of the heme-domain during incubation with chymotrypsin.

A very low amount of radioactivity in the heme-domain is also found when the residual activity of the sample is 30% and 5.5 residues are modified. In this case 43%, or 31% of the radioactivity is expected in the heme-domain if the essential residue is in the heme-, or in the molybdenum-domain, respectively.

The distribution of ^3H -radioactivity as shown in table 7 suggests that the single fast reacting carboxyl group is located in the heme-domain of sulfite oxidase, the essential residue however seems to be in the molybdenum-domain.

3.7. MODIFICATION OF HISTIDINE RESIDUES BY DEP

3.7.1. Selectivity of the Reagent

Modification of histidine residues of sulfite oxidase was achieved with DEP. Enzymatic activity was lost with increasing time of incubation with DEP. Even though this reagent can be expected to react fairly specifically with the side chain of histidine at pH 6, other residues may be modified, as stated by Miles (1977). Candidates for side reactions are tyrosyl, cysteinyl and lysyl residues,

Three observations strengthen the assumption that the modification of histidine residues is responsible for the inactivation of sulfite oxidase by DEP (figure 22).

(1) The difference spectrum of modified minus native sulfite oxidase was featureless in the 280 nm region, where modification of phenolic groups would yield a change of absorption (not shown).

(2) The enzymatic activity could be restored by incubating the modified enzyme with hydroxylamine as shown in figure 21. Removal of the carbethoxy group from other residues than histidine is either slow (tyrosine) or not possible (cysteine and lysine) (Miles, 1977).

(3) Incubation of sulfite oxidase with 5,5'-dithiobis-(2-nitrobenzoic acid), a reagent for sulphydryl group modification, did not affect the activity of the enzyme (not shown).

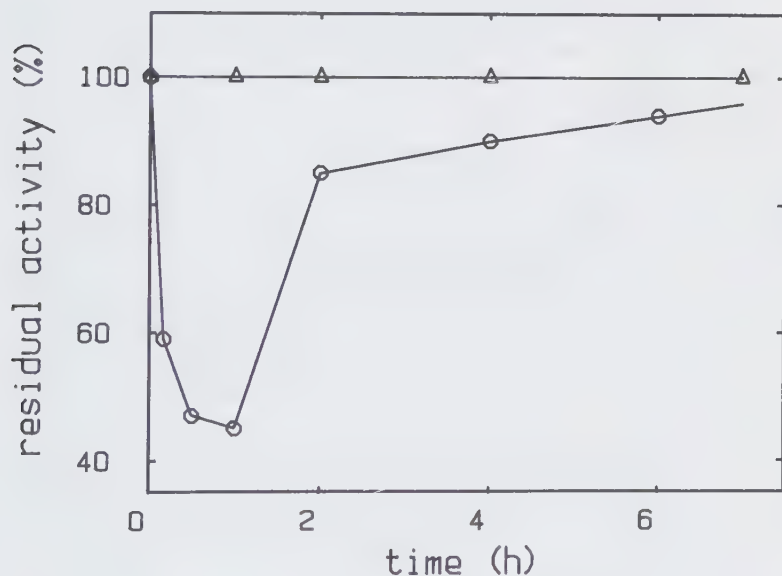


Figure 21: Reactivation of sulfite oxidase inactivated by DEP

Sulfite oxidase (21 μ M) in 0.1 M potassium phosphate, pH 6.0 was treated with 1 mM DEP for 1 hour at 20°C. Then the reaction mixture was cooled to 4°C and hydroxylamine was added to a final concentration of 0.1 M. Samples were withdrawn, separated from reagent by gel filtration and assayed in the cytochrome c assay. In a control experiment (Δ) DEP was omitted.

3.7.2. Effect of Cytochrome c

Cytochrome c is the physiological electron acceptor of sulfite oxidase. The rate of inactivation of the enzyme by modification of an essential residue in presence of its reaction partner will be decreased, if the critical residue is at or near the binding site for cytochrome c.

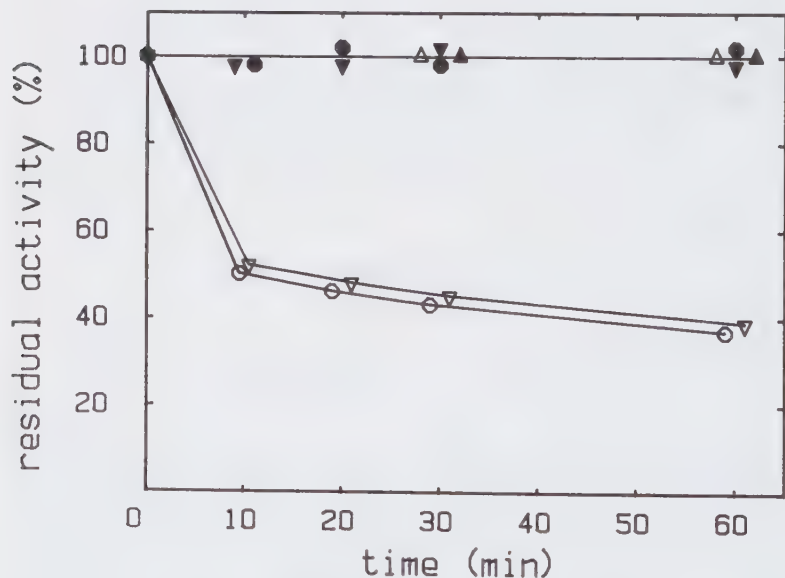


Figure 22: Inactivation of sulfite oxidase by DEP in presence and absence of cytochrome c

20 μ M sulfite oxidase with (▽) or without (○) 100 μ M cytochrome c in 10 mM potassium phosphate, pH 6.0 was incubated with 1 mM DEP. Aliquots were withdrawn at the time points indicated and immediately assayed for cytochrome c reductase activity (open symbols) and ferricyanide reductase activity (closed symbols). In a control containing cytochrome c, DEP was omitted (Δ).

When sulfite oxidase was incubated with DEP in presence of cytochrome c, the rate of inactivation was not affected, even under the conditions shown in figure 22 where, on average, 90% of the enzyme was saturated with cytochrome c. This result indicates that the crucial histidine was equally accessible for DEP in the enzyme-substrate complex and in the enzyme alone. The critical residue(s) seems therefore not to be at, or near to the binding site of cytochrome c.

3.7.3. Incorporation of DEP and its Influence on Activity and Kinetic Constants of Sulfite Oxidase

Imidazole side chains of histidines were modified with DEP to the N-carbethoxy derivatives as shown by Miles (1977). The number of residues modified was easily estimated spectroscopically. As depicted in figure 23 the cytochrome c reductase activity of sulfite oxidase decreased when the number of modified residues increased. The ferricyanide reductase activity however remained unaffected. DEP was added in portions because the reagent is quickly hydrolysed in phosphate buffer (Berger, 1975). About 6 histidine residues per enzyme monomer are modified when the residual activity was still about 40%.

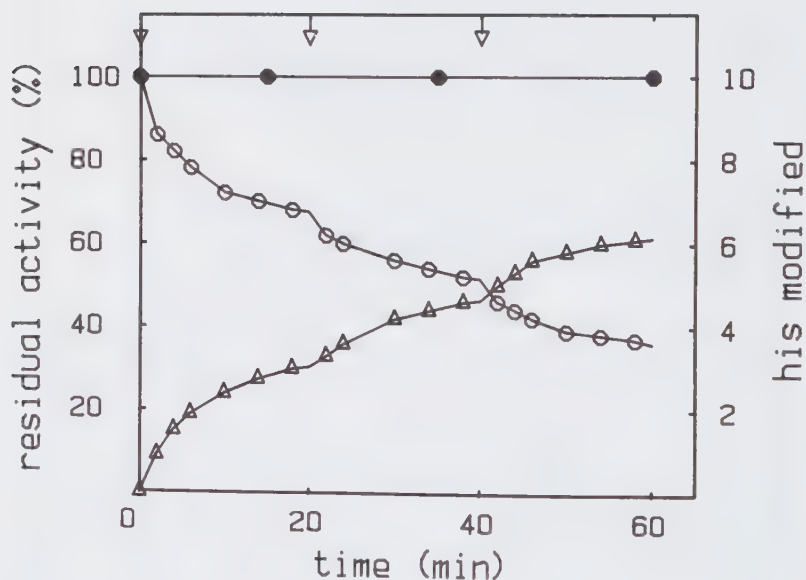


Figure 23: Inactivation of sulfite oxidase by DEP

A solution (640 μ l) of 11 μ M sulfite oxidase in 10 mM potassium phosphate, pH 6.0 was treated with 2 x 5 μ l and 1 x 10 μ l of 25 mM DEP added at the time points indicated by arrows. Samples were withdrawn and assayed by the cytochrome c assay (○) and by the ferricyanide assay (●). The number of histidines modified per mole of sulfite oxidase monomer was determined (Δ) from the change of absorbance at 240 nm according to Miles (1977).

The relationship between loss of enzymatic activity and the number of residues modified is shown in figure 24. In panel A, the results of figure 23 were plotted according to the equation $a^{1/i} = 1 - m / p$ where a is the fraction of residual activity, m is the number of groups modified, and p is the number of equally reactive groups among which there are i essential residues (Tsou, 1962). For the data shown in panel A, i obtained from a double logarithmic plot was 0.98, and p , determined by a least squares fit, was 9.75.

Panel B of figure 24 shows a different experiment which led to the same result: equal amounts of sulfite oxidase were treated with increasing amounts of DEP for 20 minutes. The solid line shown in this figure was plotted for $p = 10$ and $i = 1$. For comparison, calculated lines are shown for $p = 10$, $i = 2$, and $p = 10$, $i = 3$.

Both experiments shown in figure 24 strongly indicate that out of the 25 1 histidine residues of the sulfite oxidase dimer (Kessler & Rajagopalan, 1972) ten were equally reactive in each monomer and about two were non-reactive. Among those ten equally reactive groups a single histidine is crucial for the cytochrome c reductase activity, but not for the ferricyanide reductase activity.

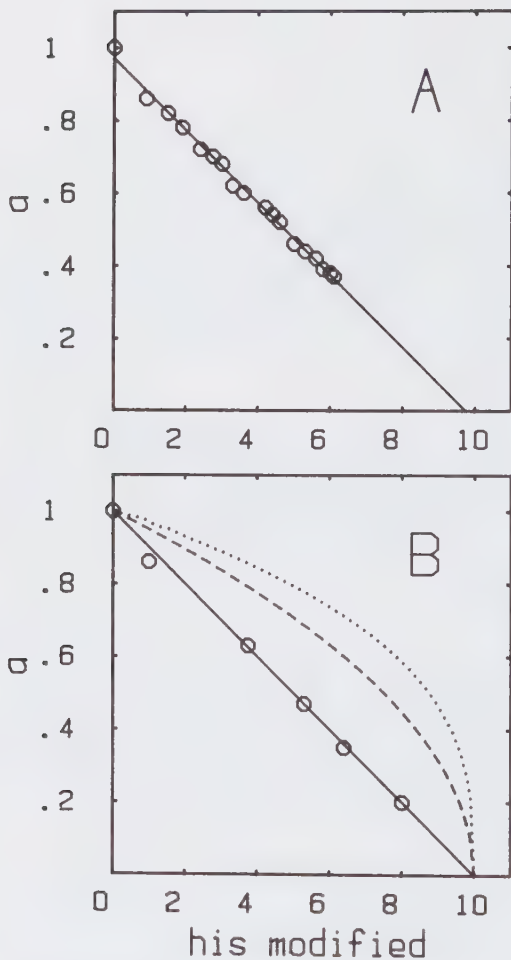


Figure 24: Relationship between residual enzyme activity and the number of histidine residues modified

Panel A: The data from figure 23 are plotted according to the equation $a^{1/i} = 1 - m/p$. The solid line shows a least squares fit which gave $i = 0.98$ and $p = 9.75$.

Panel B: Five samples of $20 \mu M$ sulfite oxidase in $0.1 M$ potassium phosphate, pH 6.0 were treated with 0.125, 0.5, 1.25, 2.5 and 5 mM DEP, respectively, for 20 minutes at $20^\circ C$. Probes were assayed for cytochrome c reductase activity and the number of histidines modified was determined. The solid line was calculated for $i = 1$ and $p = 10$. For comparison, calculated lines for $p = 10$ and $i = 2$ (-----) and $i = 3$ (.....) are shown.

The kinetic constants of probes with different degrees of residual activity in the cytochrome c assay after modification with DEP are shown in table 8. The values of K_m and V_{max} indicate that the electron transfer and the binding of cytochrome c are affected. The presence of cytochrome c during modification does not alter the result. This observation indicates that modification at the cytochrome c binding site is not responsible for the decrease in K_m . The essential histidine residue seems to be important for the intramolecular or the intermolecular electron transfer.

Table 8: Kinetic constants of sulfite oxidase modified by DEP

modification conditions	% resid. activ.		
	cytochrome c	K_m	V_{max}
	assay	(μM)	(s^{-1})
20 μM sulfite oxidase modified			
at 20°C in 10 mM potassium			
phosphate, pH 6.0			
control without DEP	100	0.94	80
1 mM DEP for 30 minutes	45	0.42	32
1 mM DEP for 30 minutes			
in presence of 100 μM cyt c	45	0.45	28
2 x 1 mM DEP, 30 minutes each	20	0.32	20

4. DISCUSSION

4.1. BINDING OF CYTOCHROME c TO SULFITE OXIDASE

The binding study with chicken liver sulfite oxidase and horse heart cytochrome c revealed two binding sites which are equal and noninteracting and have a dissociation constant of $7.5 \mu\text{M}$ at an ionic strength of 10 mM.

Two equal and noninteracting binding sites were also found for the complex of beef sulfite oxidase with horse cytochrome c. The dissociation constant, however, was much smaller, $0.1 \mu\text{M}$ at 10 mM ionic strength (Speck et al., 1981).

Binding of two molecules of cytochrome c per enzyme monomer has also been shown for cytochrome c oxidase (Rieder & Bosshard, 1978) and for cytochrome c reductase (Bosshard et al., 1979), but the two binding sites have very different affinities in both cases.

The significance of the two binding sites on sulfite oxidase is not clear. The binding of horse cytochrome c to sulfite oxidase is much weaker than binding to other physiological reaction partners.

The binding site on cytochrome c was found to be the same for all its reaction partners (Rieder & Bosshard, 1980; Margolias & Bosshard, 1983). A group of positively charged lysyl residues of cytochrome c are thought to make contact with negatively charged carboxyl groups of the reaction partners.

In the case of cytochrome c peroxidase from baker's yeast, the molecular surface recognized by cytochrome c has been mapped by differential chemical modification (Bechtold & Bosshard, 1985) and by covalent cross-linking (Waldmeyer & Bosshard, 1985). A set of suitably located carboxyl groups was found to make contact with positively charged lysine residues of cytochrome c. These experimental results support the prediction of computer-aided model building (Poulos & Kraut, 1980; Finzel et al., 1984).

A similar structure for the heme-domain of sulfite oxidase and cytochrome b_5 was proposed by Guiard & Lederer (1979a). In a hypothetical electron transfer complex of cytochrome c and cytochrome b_5 , Salemm (1976) suggested four lysines of cytochrome c to juxtapose with four acidic groups of cytochrome b_5 . Only two of these acidic groups are conserved in chicken sulfite oxidase, as shown in figure 25. There seem to be too few suitably located acidic groups in the heme-domain of chicken sulfite oxidase for the type of tight binding of cytochrome c as discussed by Guiard & Lederer (1979a). The relatively high dissociation constant of the complex of horse cytochrome c with chicken sulfite oxidase may be explained by a paucity of ionic bonds.

4.2. THE REACTION OF SULFITE OXIDASE WITH CYTOCHROME c

The steady-state kinetic constants for the reaction of chicken sulfite oxidase with horse cytochrome c in 50 mM Tris-HCl, pH 7.5 at 20°C were $K_m = 0.9 \mu M$ and $V_{max} = 85 \text{ sec}^{-1}$. The latter is a rather slow turnover number as compared to the reaction of cytochrome c with purified cytochrome c reductase, which under similar conditions yields maximal turnover numbers ranging from about 500 to 800 sec^{-1} (Kang et al., 1978).

The small turnover number for the reaction of sulfite oxidase with cytochrome c seems not to be due to a slow rate of dissociation of ferri-cytochrome c from the enzyme, since sulfite oxidase binds only weakly to cytochrome c; quite in contrast to the reaction of cytochrome c with oxidase where product dissociation is rate limiting under most experimental conditions (Smith et al., 1979; Rosevear et al., 1980).

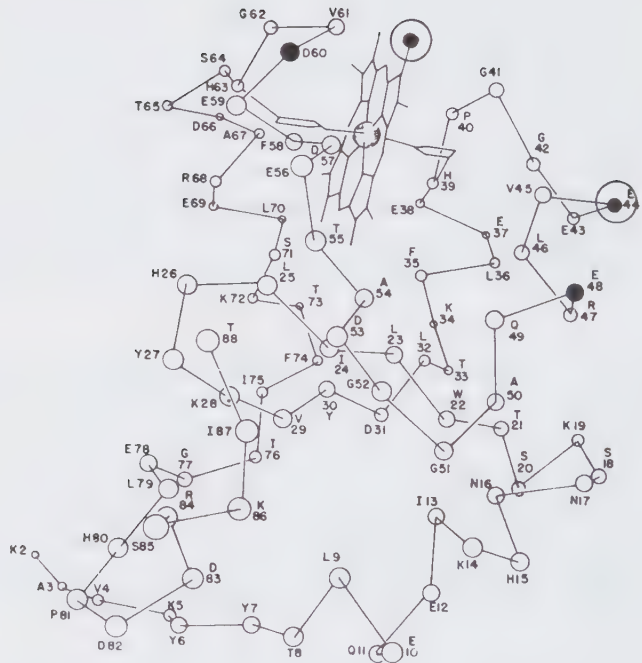


Figure 25: Acidic groups of cytochrome b_5 interacting with cytochrome c

Filled circles show the four acidic groups on cytochrome b_5 (Asp 44, 48, 60, and the most exposed heme propionate) proposed to interact with cytochrome c by Salemme (1976). Two of the four acidic groups (Asp 44 and the heme propionate; shown by a circle around their position) are conserved in sulfite oxidase from chicken liver (Guiard & Lederer, 1979).

For beef sulfite oxidase Speck et al. (1981) measured a Michaelis constant of $0.36 \mu\text{M}$ and a maximal turnover number of approximately 50 sec^{-1} in 50 mM Tris-HCl, pH 8.0. These values are not very far from those determined for the chicken enzyme. The difference in K_m is easily explained by the weaker binding of cytochrome c to the chicken enzyme.

The apparent Michaelis constant is smaller than the dissociation constant determined by gel filtration. This discrepancy may be explained by a rate limiting step prior to electron transfer from heme b to cytochrome c (Speck et al., 1981).

4.3. THE ROLE OF CARBOXYL GROUPS FOR THE ENZYMATIC ACTIVITY OF SULFITE OXIDASE

Carbodiimides were found to be suitable reagents for the determination of the role of carboxyl groups for the activity of sulfite oxidase. Modification under mild conditions is possible, and therefore protein denaturation can be ruled out as a cause for inactivation. Oxidation of sulfite at the molybdenum-center is not affected by the reagent, as shown by the unimpaired ferricyanide reductase activity (see figure 3). This result indicates that inactivation by carbodiimide interferes with electron transfer from the molybdenum-center to heme b and/or the transfer from heme b to heme c.

Two questions arise. (1) Is the inactivation of sulfite oxidase caused by the chemical modification of one or very few essential groups or rather by the modification of several carboxyl groups none of which can be regarded as essential in a strict sense? A residue is called essential in a strict sense if its modification fully inhibits enzyme activity. (2) Where are the carboxyl groups located whose modification inactivates the enzyme?

The inactivation reaction was first order. This result is compatible with the assumption of a single essential carboxyl group or with a series of carboxyls that all react at the same rate and among which there is

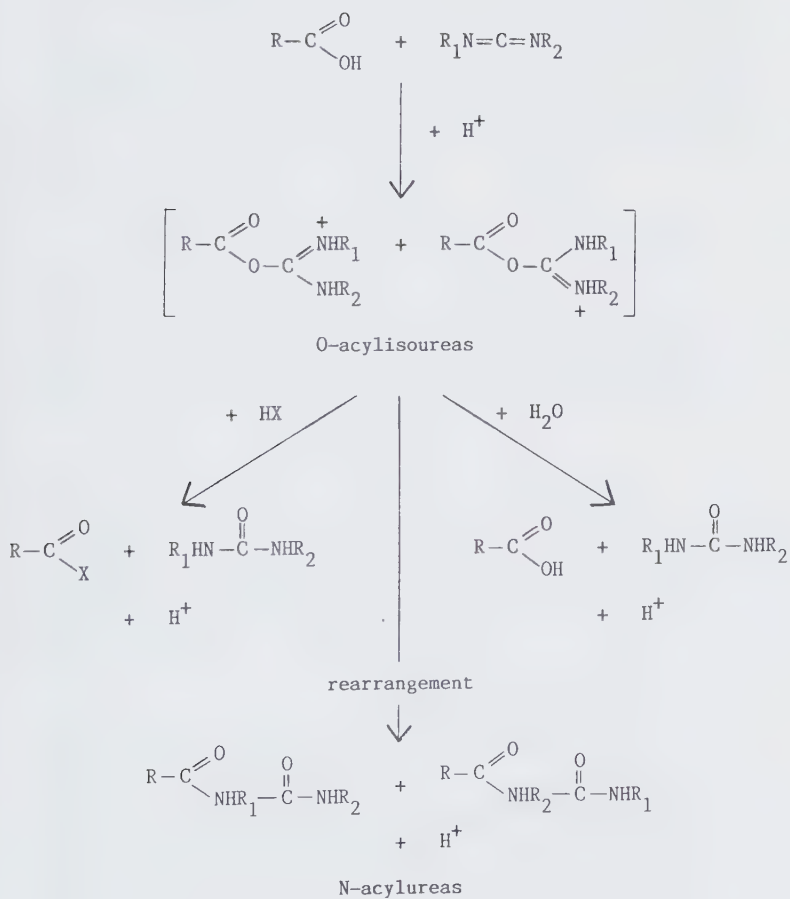
one or more essential residue. The first order kinetic of the modification reaction is equally compatible with the concept of several carboxyls that are not strictly essential, provided that all these residues are reacting at the same rate.

The pathway of the reaction of a carboxyl group with a carbodiimide is shown in scheme 1. The first step of the reaction is the formation of unstable O-acylisourea derivatives. Three reactions are now possible.

The carboxyl group can be regenerated through a nucleophilic attack by water with the conversion of one molecule of carbodiimide to the corresponding urea. This reaction simulates the formation of a reversible enzyme-reagent complex prior to irreversible inactivation as indicated by figure 19 B in section 3.6.4.

The O-acylisoureas can rearrange to form stable N-acylureas via an intramolecular acyl transfer. Some acidic side chains were found to be especially reactive by this pathway (Timkovich, 1977). The most likely reason is that once the activated O-acylisourea is formed, the bulk of the protein plus the substituents of the urea sterically block the attack of water and other nucleophiles. The only open pathway, or at least the one now favored, is internal rearrangement. The detection of the N-acylureas is possible with ^3H -labeled carbodiimide.

In the presence of a strong nucleophile other than water, the O-acylisourea derivatives react to an amidated product plus the urea derived from the carbodiimide. The formation of the amidated derivative of the carboxyl group can be estimated by amino acid analysis or by determination of the radioactivity incorporated if a labeled nucleophile is used. In case the attacking nucleophile is a lysine side chain or an N-terminal amino group of the protein, inter- or intra-molecular cross-linking occurs.



Scheme 1: Modification of carboxyl groups by carbodiimides

In the present case enzyme inactivation occurs in the absence of an added nucleophile, inactivation must be due to the formation of stable N-acylurea derivatives. This conclusion is supported by two observations. First, the time course of inactivation is the same in the presence of excess taurine which might scavenge the O-acylisoureas and lead to amidated carboxyl groups. Only 1.2 mol of [^3H]taurine per enzyme monomer is incorporated when the enzyme has lost 80% of the activity. This result contrasts that for cytochrome c peroxidase (Waldmeyer et al., 1982). Second, no cross-linking of cytochrome c with sulfite oxidase and no cross-linked oligomeric enzyme species are being formed.

Quantification of N-acylurea derivatives is difficult without radioactively labeled carbodiimide. But labeled carbodiimide is not commercially available at an affordable price. [^3H]-CMC was therefore synthesized following published procedures for use in the modification reaction.

The result of the incorporation study shows clearly that about seven residues are modified when the enzyme shows still 20% of residual activity. The rearrangement of the O-acylisourea to the stable N-acylurea is obviously much faster than the reaction with the nucleophile, even if the nucleophile is present in great excess. The present example shows once more that it is dangerous to deduce the number of modified carboxyl groups from the number of amidated residues.

If the incorporation of ^3H -radioactivity in relation to the loss of enzymatic activity is analyzed according to Tsou (1962) it is possible to show that modification by [^3H]-CMC of one carboxyl group, or at the most of two groups, is responsible for inactivation of sulfite oxidase.

This finding does not agree with the concept that for proper binding of cytochrome c several acidic groups are important, of which none can be said to be essential in the strict sense. This concept forms the basis of hypothetical models for the interaction of cytochrome c with cytochrome b_5 (Salemme, 1976) and cytochrome c peroxidase (Poulos & Kraut, 1980). The model for the peroxidase was supported by protein-chemical experiments (Waldmeyer et al., 1982; Waldmeyer & Bosshard, 1985; Bechtold & Bosshard, 1985).

As discussed in section 4.1., there seem to be too few suitably located carboxyl groups in the heme-domain of sulfite oxidase for the type of ionic interactions proposed for the cytochrome b_5 -cytochrome c or the cytochrome c peroxidase-cytochrome c complex. This fact might also be the reason for the absence of cross-linking between cytochrome c and sulfite oxidase by a carbodiimide at conditions where about 40% of a covalent complex was formed between cytochrome c peroxidase and cytochrome c.

The residue which was found to be essential for activity seems not to be at, or near to the binding site of cytochrome c for three reasons: Modification in presence of cytochrome c did not protect the enzyme from inactivation, the Michaelis-constant of the reaction of sulfite oxidase with cytochrome c is not very much affected by the modification of the enzyme, and no covalent cross-linking of sulfite oxidase and cytochrome c by a carbodiimide was observed.

The distribution of radioactivity incorporated at different degrees of modification may be taken to indicate that the essential residue(s) is located on the molybdenum-domain. It could be involved in the intramolecular electron transfer to heme b.

4.4. THE ROLE OF HISTIDINE RESIDUES FOR THE ENZYMATIC ACTIVITY OF SULFITE OXIDASE

Inactivation of sulfite oxidase by diethylpyrocarbonate (DEP) did lead to modification of ten histidine residues per monomer. All of these residues were equally reactive. 25 ± 1 histidine residues were found for the chicken sulfite oxidase dimer by Kessler & Rajagopalan (1972). This number indicates, that beside the ten equally reactive groups, there are about two non-reactive ones in each monomer.

One imidazol group per monomer was found to be essential for activity. This result is similar to that obtained with the heme enzyme cytochrome c peroxidase by Bosshard et al. (1984). In that case, modification of a single histidine residue abolished the reaction of the enzyme with H_2O_2 . In both cases, the loss of activity occurred in presence and absence of cytochrome c. The crucial histidine residue of cytochrome c peroxidase is located in the heme pocket, but it is not a heme ligand.

From our data it was not possible to decide whether the crucial histidine belongs to the heme-domain of sulfite oxidase, although this seems a reasonable guess because electron transfer from the molybdenum-center to ferricyanide was uninhibited.

Sequence comparison among the few members of the cytochrome b_5 family reveals that only three histidine residues have been conserved. In the heme-domain of sulfite oxidase they are the heme ligands His 40 (His 39 in b_5) and His 65 (His 63 in b_5), and His 15 which is on the surface of the molecule, far from the heme pocket as shown in figure 26 (Guiard & Lederer, 1979).

If the two heme ligands of sulfite oxidase are held as rigidly in place as those of cytochrome b_5 (Matthews et al., 1972) they may constitute the two non-reactive histidines of sulfite oxidase.

The only other two histidines of the heme-domain of sulfite oxidase are non-conserved and lay to the left of the heme crevice in an area of high sequence variation. Both of them lay near the proposed binding site of cytochrome c on cytochrome b_5 (Salemme, 1976) and could play an important role in the intra- or intermolecular electron transfer reaction.

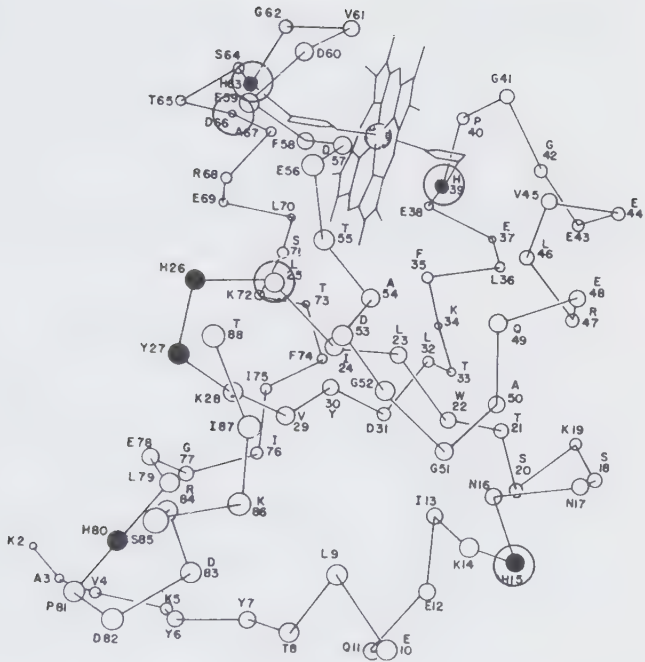


Figure 26: Histidine residues of cytochrome b_5 and chicken sulfite oxidase heme-domain

The histidine residues of chicken cytochrome b_5 and chicken sulfite oxidase heme-domain are shown in the three dimensional model of calf cytochrome b_5 . Filled circles show the histidine residues of chicken cytochrome b_5 . The residues of chicken sulfite oxidase are shown by a circle around their position.

4.5. CONCLUSIONS

Modification of one or two carboxyl groups and a single histidine residue of sulfite oxidase specifically inhibits the electron transfer reaction of the enzyme with its physiological electron acceptor cytochrome c. Oxidation of sulfite at the molybdenum-center followed by electron transfer to the artificial acceptor ferricyanide is not inhibited. Hence, the chemical modification interferes with some step(s) after the oxidation of sulfite by the molybdenum-center, either with the intramolecular electron transfer to heme b, or with the intermolecular one to cytochrome c.

The finding of one or two carboxyl groups essential for the enzymatic activity comes as a surprise. All of the reaction partners of cytochrome c that have been investigated so far were found to contain several acidic residues that are important for binding of cytochrome c through ionic bonds. This has been shown for cytochrome c₁, subunit VIII of ubiquinone-cytochrome c oxidoreductase, subunit II of cytochrome oxidase, cytochrome b₅, cytochrome c peroxidase, plastocyanin and adrenodoxin (Gutweniger et al., 1983; Millett et al., 1983; Dailey & Strittmatter, 1979; Bechtold & Bosshard, 1985; Geren et al., 1983; Geren et al., 1984). But none of these carboxyl groups is thought to be essential in the strict sense.

The role of the acidic group(s) found to be essential for sulfite oxidase activity must be different. If the essential group is on the molybdenum-domain involvement in the intramolecular electron transfer reaction seems likely.

The course of inactivation of sulfite oxidase with DEP is surprisingly similar to that observed with cytochrome c peroxidase (Bosshard et al., 1984), even though the two enzymes are structurally unrelated. In both cases one single histidine was found to be essential for the enzymatic activity. Modification of this residue was not inhibited by the presence of the physiological reaction partner cytochrome c.

The crucial histidine of cytochrome c peroxidase was found to be necessary for the formation of a reaction intermediate with H_2O_2 . The actual function of the essential histidine of sulfite oxidase remains to be determined.

REFERENCES

- ALAIRE Y. C., ULRICH C. E., BUSEY W. M., KRUMM A. A. & MACFARLAND H. N. (1972) Long-term continuous exposure to sulfur dioxide in cynomolgus monkeys.
Arch. Environ. Health, 24, 115-128.
- ALARIE Y. C., KRUMM A. A., BUSEY W. M., ULRICH C. E. & KANTZ R. J. (1975) Long-term exposure to sulfur dioxide, sulfuric acid mist, fly ash, and their mixtures.
Arch. Environ. Health, 30, 254-262.
- AMDUR M. O. (1971) Aerosols formed by the oxidation of sulfur dioxide.
Arch. Environ. Health, 23, 459-468.
- ARIKI M. & FUKUI T. (1978) Modification of rabbit muscle phosphorylase b by a water-soluble carbodiimide.
J. Biochem, 83, 183-190.
- ARRIGONI O. (1959) The enzymatic oxidation of sulphite in mitochondrial preparations of pea internodes.
The Italian J. Biochem, VIII, 181-186.
- BALCHUM O. J. (1959) Absorption and distribution of $^{35}\text{SO}_2$ inhaled through the nose and mouth of dogs.
Am. J. Physiol, 197, 1317-1321.
- BANKS T. E., BLOSSEY B. K. & SHAFER J. A. (1969) Inactivation of α -chymotrypsin by a water-soluble carbodiimide.
J. Biol. Chem, 244, 6323-6333.
- BECHTOLD R. & BOSSHARD H. R. (1985) Structure of an electron transfer complex. Chemical modification of carboxyl groups of cytochrome c peroxidase in presence and absence of cytochrome c.
J. Biol. Chem, 260, 5191-5200.

- BERGER S. (1975) Diethyl pyrocarbonate: An examination of its properties in buffered solutions with a new assay technique.
Anal. Biochem. 67, 428-437.
- BOSSHARD H. R., ZUERRER M., SCHAEGER H. & VON JAGOW G. (1979) Binding of cytochrome c to the cytochrome bc_1 complex (complex III) and its subunits cytochrome c_1 and b.
Biochem. Biophys. Res. Commun. 89, 250-258.
- BOSSHARD H. R., BAENZIGER J., HASLER T. & POULOS T. L. (1984) The cytochrome c peroxidase-cytochrome c electron transfer complex. The role of histidine residues.
J. Biol. Chem. 259, 5683-5690.
- BRADFORD M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding.
Anal. Biochem. 72, 248-254.
- CALABRESE E., SACCO C., MOORE G. & DINARDI S. (1981) Sulfite oxidase deficiency: A high risk factor in SO_2 , sulfite, and bisulfite toxicity?
Medical Hypotheses 7, 133-145.
- CARRAWAY K. L. & KOSHLAND D. E. Jr. (1968) Reaction of tyrosine residues in proteins with carbodiimide reagents.
Biochim. Biophys. Acta 160, 272-274.
- CARRAWAY K. L. & TRIPLETT R. B. (1970) Reaction of carbodiimides with protein sulfhydryl groups.
Biochim. Biophys. Acta 200, 564-566.
- CHARLES A. M. (1969) Mechanism of thiosulfate oxidation by *Thiobacillus intermedius*.
Arch. Biochem. Biophys. 129, 124-130.
- CHARLES A. M. & SUZUKI I. (1966) Purification and properties of sulfite: cytochrome c oxidoreductase from *Thiobacillus novellus*.
Biochim. Biophys. Acta 128, 522-534.

- COHEN H. J. & FRIDOVICH I. (1971) Hepatic sulfite oxidase. Purification and properties.
J. Biol. Chem. 246, 359-366.
- COHEN H. J. & FRIDOVICH I. (1971a) Hepatic sulfite oxidase. The nature and function of the heme prosthetic group.
J. Biol. Chem. 246, 367-373.
- COHEN H. J., FRIDOVICH I. & RAJAGOPALAN K. V. (1971) Hepatic sulfite oxidase. A functional role of molybdenum.
J. Biol. Chem. 246, 374-382.
- COHEN H. J., DREW R. T., JOHNSON J. L. & RAJAGOPALAN K. V. (1973) Molecular basis of the biological function of molybdenum. The relationship between sulfite oxidase and the acute toxicity of bisulfite and SO_2 .
Proc. Nat. Acad. Sci. 70, 3655-3659.
- DAILEY H. A. & STRITTMATTER P. (1979) Modification and identification of cytochrome b_5 carboxyl groups involved in protein-protein interaction with cytochrome b_5 reductase.
J. Biol. Chem. 254, 5388-5396.
- DURAN M., KORTELAND J., BEEMER F. A., vd HEIDEN C., deBREE P. K. & BRINK M. (1979) Variability of sulfitoria: combined deficiency of sulfite oxidase and xanthine oxidase.
In Models of the studies of inborn errors of metabolism (Hommes ed.), Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 103-107.
- FERGUSON-MILLER S., BRAUTIGAN D. L. & MARGOLIASH E. (1976) Correlation of the kinetics of electron transfer activity of various eukaryotic cytochromes c with binding to mitochondrial cytochrome c oxidase.
J. Biol. Chem. 251, 1104-1115.
- FINZEL B. C., POULOS T. L. & KRAUT J. (1984) Crystal structure of yeast cytochrome c peroxidase refined at 1.7 Å resolution.
J. Biol. Chem. 259, 13027-13036.

- FROMAGEOT P., VAILLANT R. & PEREZ-MILAN H. (1960) Oxydation du sulfite en sulfate par la racine d'avoine.
Biochim. Biophys. Acta 44, 77-85.
- FUKUSHIMA K., ITO A., OMURA T. & SATO R. (1972) Occurrence of different types of cytochrome b_5 -like hemoprotein in liver mitochondria and their intramitochondrial location.
J. Biochem. 71, 447-461.
- GEREN L. M., STONEHUENER J., DAVIS J. D. & MILLETT F. (1983) The use of a water-soluble carbodiimide to cross-link cytochrome c to plastocyanin.
Biochim. Biophys. Acta 724, 62-68.
- GEREN L. M., O'BRIEN P., STONEHUERNER J. & MILLET F. (1984) Identification of specific carboxylate groups on adrenodoxin that are involved in the interaction with adrenodoxin reductase.
J. Biol. Chem. 259, 2155-2160.
- GIBSON W. B. & STRONG F. M. (1973) Metabolism and elimination of sulfite by rats, mice and monkeys.
Fd. Cosmet. Toxicol. 11, 185-198.
- GOTO M., SAKURAI A., OHTA K. & YAMALAMI H. (1969) Die Struktur des Urothions.
J. Biochem. 65, 611-620.
- GUIARD B. & LEDERER F. (1977) The " b_5 -like" domain from chicken liver sulfite oxidase: A new case of common ancestral origin with liver cytochrome b_5 and baker's yeast cytochrome b_2 core.
Eur. J. Biochem. 74, 181-190.
- GUIARD B. & LEDERER F. (1979) Amino acid sequence of the ' b_5 -like' heme-binding domain from chicken sulfite oxidase.
Eur. J. Biochem. 100, 441-453.

- GUIARD B. & LEDERER F. (1979a) The "cytochrome b_5 fold": Structure of a novel protein superfamily.
J. Mol. Biol. 135, 639-650.
- GUNNISON A. F., FARRUGELLA T. J., CHIANG G., DULAK L., ZACHARDI J. & BIRKNER J. (1981) Sulfite toxicity: A critical review of in vitro and in vivo data.
Fd. Cosmet. Toxicol. 19, 209-220.
- GUTWENIGER H. E., GRASSI C. & BISSON R. (1983) Interaction between cytochrome c and ubiquinone-cytochrome c oxidoreductase: A study with water-soluble carbodiimides.
Biochem. Biophys. Res. Commun. 116, 272-283.
- HEIMBERG M., FRIDOVICH I. & HANDLER P. (1953) The enzymatic oxidation of sulfite.
J. Biol. Chem. 204, 913-926.
- HELMERHORST E. & STOKES B. G. (1980) Microcentrifuge desalting: A rapid, quantitative method for desalting small amounts of protein.
Anal. Biochem. 104, 130-135.
- HICKEY R. J., CLELLAND R. C. & BOYCE D.E. (1974) Atmospheric sulfur dioxide, nitrogen dioxide, and lead as mutagenic hazards to human health.
Mutat. Res. 26, 445-446.
- HOARE D. G. & KOSHLAND D. E. Jr. (1967) A method for the quantitative modification and estimation of carboxylic acid groups in proteins.
J. Biol. Chem. 242, 2447-2453.
- HORIIKE K. & McCORMICK D. B. (1979) Correlation between biological activity and the number of functional groups chemically modified.
J. theor. Biol. 79, 403-414.
- HUMMEL J. P. & DREYER W. J. (1962) Measurement of protein-binding phenomena by gel filtration.
Biochim. Biophys. Acta 63, 530-532.

- IRREVERRE F., MUDD S. H., HEIZER W. D. & LASTER L. (1967) Sulfite oxidase deficiency: Studies of a patient with mental retardation, dislocated ocular lens, and abnormal urinary excretion of S-sulfo-L-cysteine, sulfite and thiosulfate.
Biochem. Med. 1, 187-217.
- ITO A. (1971) Hepatic sulfite oxidase identified as cytochrome b_5 -like pigment extractable from mitochondria by hypotonic treatment.
J. Biochem. 70, 1061-1064.
- JOHNSON J. L. & RAJAGOPALAN K. V. (1974) Effect of tungsten on xanthine oxidase and sulfite oxidase in the rat.
J. Biol. Chem. 250, 859-866.
- JOHNSON J. L. & RAJAGOPALAN K. V. (1976) Purification and properties of sulfite oxidase from human liver.
J. Clin. Invest. 58, 543-550.
- JOHNSON J. L. & RAJAGOPALAN K. V. (1977) Tryptic cleavage of rat liver sulfite oxidase. Isolation and characterisation of molybdenum and heme domain.
J. Biol. Chem. 252, 2017-2025.
- JOHNSON J. L., JONES H. P. & RAJAGOPALAN K. V. (1977) In vitro reconstitution of demolybdosulfite oxidase by a molybdenum cofactor from rat liver and other sources.
J. Biol. Chem. 252, 4994-5003.
- JOHNSON J. L. & RAJAGOPALAN K. V. (1982) Structural and metabolic relationship between the molybdenum cofactor and urothione.
Proc. Natl. Acad. Sci. 79, 6856-6860.
- JOSHI V. C., RAMAKRISHNA KURUP C. K. & RAMASARMA T. (1969) The cryptic nature of hepatic microsomal sulfite oxidase.
Biochem. J. 111, 297-301.

- KAGEDAL B., KAELLBERG M. & SOERBO B. (1986) A possible involvement of glutathione in the detoxication of sulfite.
Biochem. Biophys. Res. Commun. 136, 1036-1041.
- KANG C. H., BRAUTIGAN D. L., OSHEROFF N. & MARGOLIASH E. (1978) Definition of cytochrome c binding domains by chemical modification. Reaction of carboxydinitrophenyl- and trinitrophenyl-cytochromes c with baker's yeast cytochrome c peroxidase.
J. Biol. Chem. 253, 6502-6510.
- KESSLER D. L. & RAJAGOPALAN K. V. (1972) Purification and properties of sulfite oxidase from chicken liver. Presence of molybdenum in sulfite oxidase from diverse sources.
J. Biol. Chem. 247, 6566-6573.
- KESSLER D. L. & RAJAGOPALAN K. V. (1974) Hepatic sulfite oxidase. Effects of anions on interaction with cytochrome c.
Biochim. Biophys. Acta 370, 389-398.
- KITZ R. & WILSON I. B. (1962) Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase.
J. Biol. Chem. 237, 3245-3249.
- KOPPENOL W. H. & MARGOLIASH E. (1982) The asymmetric distribution of charges on the surface of horse cytochrome c. Functional implications.
J. Biol. Chem. 257, 4426-4437.
- KUWAHARA T., YOSHIMOTO I. & ITO A. (1982) Comparative immunochemical studies of sulfite oxidases of vertebrate livers.
J. Biochem. 92, 1925-1931.
- LAEMMLI U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T₄.
Nature (London) 227, 680-685.

- LYRIC R. M. & SUZUKI I. (1970) Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioautotrophicus*. Survey of enzymes and properties of sulfite: cytochrome c oxidoreductase.
Can. J. Biochem. 48, 334-343.
- MacLEOD R. M., FARKAS W., FRIDOVICH I. & HANDLER P. (1961) Purification and properties of hepatic sulfite oxidase.
J. Biol. Chem. 236, 1841-1846.
- MARGOLIAS E. & BOSSHARD H. R. (1983) Guided by electrostatics, a textbook protein comes of age.
Trends Biochem. Sci. 8, 316-320.
- MATHEWS F. S., LEVINE M. & ARGOS P. (1972) Three-dimensional fourier synthesis of calf liver cytochrome b_5 at 2.8 Å resolution.
J. Mol. Biol. 64, 449-464.
- METZ D. H. & BROWN G. L. (1969) The investigation of nucleic acid secondary structure by means of chemical modification with a carbodiimide reagent. The reaction between N-cyclohexyl-N'-beta-(4-methylmorpholinium) ethylcarbodiimide and model nucleotides.
Biochemistry 8, 2312-2328.
- MICHEL B. (1987) PhD thesis, in preparation.
- MILES E. W. (1977) Modification of histidyl residues in proteins by diethylpyrocarbonate.
Meth. Enzymol. 47, 431-442.
- MILLETT F., DE JONG C., PAULSON L. & CAPALDI R. A. (1983) Identification of specific carboxylate groups on cytochrome c oxidase that are involved in binding cytochrome c.
Biochemistry 22, 546-552.
- MUDD H. S., IRREVERRE F. & LASTER L. (1967) Sulfite oxidase deficiency in man: demonstration of the enzymatic defect.
Science 156, 1599-1602.

- NOVIKOFF A. B., HECHT L. I., PODBER E. & RYAN J. (1952) Phosphatases of rat liver. The dephosphorylation of adenosinetriphosphate. *J. Biol. Chem.* 194, 153-170.
- OLNEY J. W., MISRA C. H. & DE GUBAREFF T. (1975) Cysteine-S-sulfate: Brain damaging metabolite in sulfite oxidase deficiency. *J. Neuropathol. Exp. Neurol.* 34, 167-177.
- ONO H. & ITO A. (1984) Transport of the precursor for sulfite oxidase into intermembrane space of liver mitochondria: Binding of precursor to outer mitochondrial membrane. *J. Biochem.* 95, 353-358.
- OUCHTERLONY O. (1967) Immunodiffusion and immunoelectrophoresis. In *Handbook of experimental immunology* (D. M. Weir ed.), Blackwell Scientific Publications, Oxford, pp. 655-706.
- PARKER J. C. (1969) Influence of 2,3-diphosphoglycerate metabolism on sodium potassium permeability in human red blood cells: Studies with bisulfite and other redox agents. *J. Clin. Invest.* 48, 117-125.
- PATERSON Y. & LEACH S. J. (1980) Chain fission of poly-L-glutamic acid by a water-soluble carbodiimide. *Biochem. Biophys. Res. Commun.* 95, 1722-1729.
- PERCY A. K., MUDD H. S., IRREVERRE F. & LASTER L. (1968) Sulfite oxidase deficiency: sulfite esters in tissues and urine. *Biochem. Med.* 2, 198-208.
- POULOS T. L. & KRAUT J. (1980) A hypothetical model of the cytochrome c peroxidase-cytochrome c electron transfer complex. *J. Biol. Chem.* 255, 10322-10330.
- RAJAGOPALAN K. V. (1980) Sulfite oxidase. In *Molybdenum and molybdenum containing enzymes* (Coughlan ed.), Pergamon Press, Oxford, pp. 241-272.

- RAKITZIS E. T. (1985) Kinetics of protein modification and enzyme in-activation reactions: interpretation of reaction order.
Biochem J. 231, 493-494.
- RIEDER R. & BOSSHARD H. R. (1978) The cytochrome c oxidase binding site on cytochrome c. Differential chemical modification of lysine residues in free and oxidase bound cytochrome c.
J. Biol. Chem. 253, 6045-6053.
- RIEDER R. & BOSSHARD H. R. (1980) Comparison of the binding sites on cytochrome c for cytochrome c oxidase, cytochrome bc₁, and cytochrome c₁. Differential acetylation of lysyl residues in free and complexed cytochrome c.
J. Biol. Chem. 255, 4732-4739.
- ROSEVEAR P., VanAKEN T., BAXTER J. & FERGUSON-MILLER S. (1980) Alkyl glucoside detergents: A simpler synthesis and their effects on kinetic and physical properties of cytochrome c oxidase.
Biochemistry 19, 4108-4115.
- SALEMME F. R. (1976) An hypothetical structure for an intermolecular electron transfer complex of cytochromes c and b₅.
J. Mol. Biol. 102, 563-568.
- SCHMIDT C. L. A. & CLARK G. W. (1922) The fate of certain sulfur compound when fed to the dog.
J. Biol. Chem. 53, 193-207.
- SEGAL I. H. & JOHNSON M. J. (1963) Synthesis and characterization of sodium cysteine-S-sulfate monohydrate.
Anal. Biochem. 5, 330.
- SHAPIRO R. (1977) Genetic effects of bisulfite.
Mutation Res. 39, 149.
- SHAPIRO R. (1983) Genetic effects of bisulfite: Implications for environmental protection.
Basic Live Sci. 23, 35-60.

- SHAPIRO R., BRAVERMAN B., LOUIS J. B. & LEWIS R. E. (1973) Nucleic acid reactivity and conformation. II. Reaction of cytosine and uracil with sodium bisulfite.
J. Biol. Chem. 248, 4060-4064.
- SHEEHAN J. C. & HLAVKA J. J. (1956) The use of water-soluble and basic carbodiimides in peptide synthesis.
J. Org. Chem. 21, 439-441.
- SHIH V. E., ABROMS I. F., JOHNSON J. L., CARNEY M., MANDELL R., ROBB R. M., CLOHERTY J. P. & RAJAGOPALAN K. V. (1977) Sulfite oxidase deficiency. Biochemical and clinical investigations of a hereditary metabolic disorder in sulfur metabolism.
N. Engl. J. Med. 297, 1022-1028.
- SMITH L., DAVIES H. C. & NAVA M. E. (1979) Studies of the kinetics of oxidation of cytochrome c by cytochrome c oxidase: Comparison of spectrophotometric and polarographic assays.
Biochemistry 18, 3140-3146.
- SMITH M. B., STONEHUERNER J., AHMED A. F., STAUDENMAYER N. & MILLETT F. (1980) Use of specific trifluoroacetylation of lysine residues in cytochrome c to study the reaction with cytochrome b₅, cytochrome c₁, and cytochrome oxidase.
Biochim. Biophys. Acta 592, 303-313.
- SOUTHERLAND W. M. & RAJAGOPALAN K. V. (1978) Domain interactions in oxidized and reduced forms of rat liver sulfite oxidase.
J. Biol. Chem. 253, 8753-8758.
- SOUTHERLAND W. M., WINGE D. R. & RAJAGOPALAN K. V. (1978) The domains of rat liver sulfite oxidase. Proteolytic separation and characterization.
J. Biol. Chem. 253, 8747-8752.

- SPECK S. H., FERGUSON-MILLER S., OSHEROFF N. & MARGOLIASH E. (1979) Definition of cytochrome c binding domains by chemical modification: Kinetics of reaction with beef mitochondrial reductase and functional organization of the respiratory chain.
Proc. Natl. Acad. Sci. 76, 155-159.
- SPECK S. H., KOPPENOL W. H., DETHMERS J. K., OSHEROFF N. & MARGOLIASH E. (1981) Definition of cytochrome c binding domains by chemical modification. Interaction of horse cytochrome c with beef sulfite oxidase and analysis of steady state kinetics.
J. Biol. Chem. 256, 7394-7400.
- TAGER J. M. & RAUTANEN N. (1955) Sulfite oxidation by a plant mitochondrial system. Preliminary observations.
Biochim. Biophys. Acta 18, 111-121.
- TEJNAROVA I. (1977) The toxicity of sulphite anion in five species of laboratory animals.
C. Hyg. 22, 64-68.
- TEJNAROVA I. (1978) Sulfite oxidase activity in liver and kidney tissue in five laboratory animal species.
Toxicol. Appl. Pharmacol. 44, 251-256.
- THOMAS J. O. (1974) Chemical modification of proteins.
In Companion to Biochemistry (A. T. Bull et al., eds.), London, pp. 87-138.
- TIMKOVICH R. (1977) Detection of the stable addition of carbodiimide to proteins.
Anal. Biochem. 79, 135-143.
- TOPHAM C. M. (1985) Chemical modification of enzymes: reaction with an unstable inhibitor.
Biochem. J. 227, 1025-1026.

- TSOU Chen-lu (1962) Relation between modification of functional groups of proteins and their biological activity. A graphical method for the determination of the number and type of essential groups. *Scientia Sinica* 11, 1535-1558.
- WAKISAKA I. (1976) Sensory irritation by sulfite aerosols on the upper respiratory tract in mice. *Jap. J. Hyg.* 31, 347-352.
- WALDMEYER B. & BOSSHARD H. R. (1985) Structure of an electron transfer complex. Covalent cross-linking of cytochrome c peroxidase and cytochrome c. *J. Biol. Chem.* 260, 5184-5190.
- WALDMEYER B., BECHTOLD R., BOSSHARD H. R. & POULOS T. L. (1982) The cytochrome c peroxidase-cytochrome c electron transfer complex. Experimental support of a hypothetical model. *J. Biol. Chem.* 257, 6073-6076.
- WASHABAUGH W. M. & COLLINS K. C. (1983) Purification of aqueous ethylene glycol. *Anal. Biochem.* 134, 144-152.
- WATTIAUX-DE CONINCK S. & WATTIAUX R. (1971) Subcellular distribution of sulfite cytochrome c reductase in rat liver tissue. *Eur. J. Biochem.* 19, 552-556.
- WEBB M., STONEHUERNER J. & MILLETT F. (1980) The use of specific lysine modifications to locate the reaction site of cytochrome c with sulfite oxidase. *Biochim. Biophys. Acta* 593, 290-298.

WILCHEK M., MIRON T. & KOHN J. (1981) A highly sensitive colorimetric method for the determination of carbodiimides.

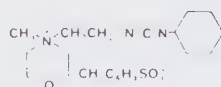
Anal. Biochem. 114, 419-421.

YOKOYAMA E., YODER R. E. & FRANK N. R. (1971) Distribution of ^{35}S in the blood and its excretion in the urine of dogs exposed to $^{35}\text{SO}_2$.

Arch. Environ. Health. 22, 389-395.

ABBREVIATIONS AND TRIVIAL NAMES

cacodylate	:	dimethylarsenic acid
CMC	:	N-cyclohexyl-N'-(2-(N-methylmorpholino)ethyl)-carbodiimide p-toluene sulfonate



[³ H]-CMC	:	N-cyclohexyl-N'-(2-(N-[³ H]methylmorpholino)ethyl)-carbodiimide p-toluene sulfonate
cyt c	:	cytochrome c
DEP	:	diethylpyrocarbonate
EDC	:	N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	:	ethylene diamine tetraacetic acid
PBS	:	phosphate buffered saline
PMSF	:	phenylmethylsulfonyl fluoride
SDS	:	sodium dodecylsulfate
SOX	:	sulfite oxidase
taurine	:	2-aminoethane sulfonic acid
Tris	:	tris(hydroxymethyl)aminomethane
Woodward's reagent K	:	N-ethyl-5-phenylisoxazolium-3'-sulfonate

SUMMARY

A basic requirement for efficient electron transfer between two hemo-proteins is their proper alignment. Binding of cytochrome c to its reaction partners is governed by ionic interactions. The same set of lysine residues on cytochrome c interacts with cytochrome c reductase, cytochrome c oxidase, cytochrome c peroxidase, and sulfite oxidase (Margoliash & Bosshard, 1983). In the case of cytochrome c oxidase (Millet et al., 1983) and cytochrome c peroxidase (Bechtold & Bosshard, 1985; Waldmeyer & Bosshard, 1985) a corresponding set of carboxyl groups was detected. No sequence homology between these two enzymes and sulfite oxidase has been found, although all of them use cytochrome c as a common physiological reaction partner.

The present study was performed to test whether the interaction between sulfite oxidase and cytochrome c follows the principles that govern the interaction with cytochrome c oxidase and cytochrome c peroxidase. In particular the role of carboxyl and imidazole groups was investigated. To this end, binding of cytochrome c to sulfite oxidase was examined by gel filtration. Chemical modification of carboxyl and imidazole groups of the enzyme was performed in presence and absence of cytochrome c and its influence on the steady state assay and on the kinetic constants of the enzymatic reaction was investigated.

The binding study with chicken sulfite oxidase and horse cytochrome c revealed two equal and non-interacting binding sites per monomer. The binding of cytochrome c to sulfite oxidase is much weaker than binding to other physiological reaction partners. The significance of the two binding sites on sulfite oxidase is not clear.

Carbodiimides were found to be suitable reagents to determine the role of carboxyl groups for the activity of sulfite oxidase, because they selectively inhibit the electron transfer from sulfite to the physiological electron acceptor cytochrome c. The reaction with the artificial acceptor ferricyanide is not affected. Hence, the chemical modification interferes with some steps after the oxidation of sulfite by the molybdenum-center of the enzyme. Inactivation was not inhibited by the presence of cytochrome c in the modification assay, indicating that the crucial residue(s) is not located at, or near to, the binding site for cytochrome c on the heme-domain of sulfite oxidase.

The time course of the inactivation reaction is compatible with the presence of one, or possibly two, essential acidic residues per enzyme monomer. With the help of radioactive carbodiimide modification of one, or at the most of two, carboxyl groups was found to be responsible for inactivation. The essential residue(s) seems to be located on the molybdenum-domain of the enzyme, away from the the binding site of cytochrome c.

This result comes as a surprise. All of the reaction partners of cytochrome c that have been investigated so far were found to contain several acidic residues that are important for binding of cytochrome c through ionic bonds, but none of them is essential in the strict sence. The role of the carboxyl group(s) found to be essential for sulfite oxidase activity seems to be another, more direct one.

Modification of imidazole groups by diethylpyrocarbonate selectively inactivates the reaction with cytochrome c. One single histidine residue was found to be essential for enzymatic activity. The inactivation was not inhibited by the presence of cytochrome c in the modification assay, indicating that the crucial residue is not located at, or near to, the binding site for cytochrome c on the heme-domain of sulfite oxidase. These results are similar to that obtained for cytochrome c peroxidase (Bosshard et al., 1984) and indicate a common role for an essential histidine residue in both enzymes even though they are structurally unrelated.

ZUSAMMENFASSUNG

Eine notwendige Voraussetzung für effiziente Elektronenübertragung zwischen Hämproteinen ist, dass sie eine spezifische Bindung eingehen können. Ionische Wechselwirkungen sind wichtig für die korrekte Bindung von Cytochrom c an seine Reaktionspartner. Ein Kranz von kationischen Lysinresten rund um die exponierte Hämkante von Cytochrom c ist beteiligt an der Bindung an Cytochrom c Reduktase, Cytochrom c Oxidase, Cytochrom c Peroxidase und Sulfioxidase (Margoliash & Bosshard, 1983). Für Cytochrom c Oxidase (Millett et al., 1983) und Cytochrom c Peroxidase (Bechtold & Bosshard, 1985; Waldmeyer & Bosshard, 1985) wurde eine komplementäre Gruppe von anionischen Carboxylresten gefunden. Zwischen diesen zwei Proteinen und Sulfioxidase besteht keine Sequenzhomologie, obwohl für alle drei Cytochrom c der physiologische Reaktionspartner ist.

Das Ziel der vorliegenden Arbeit war es, zu untersuchen, ob die Bindung von Cytochrom c an Sulfioxidase nach dem gleichen Prinzip verläuft, wie die Bindung an Cytochrom c Oxidase und Cytochrom c Peroxidase. Dazu wurde vorallem die Bedeutung von Imidazol- und Carboxylresten untersucht. Die Bindung von Cytochrom c an Sulfioxidase wurde durch Gelfiltration bestimmt. Carboxyl- oder Imidazolreste wurden in An- und Abwesenheit von Cytochrom c chemisch modifiziert. Der Einfluss der Modifikation auf die Reaktion im Fließgleichgewicht und auf die kinetischen Konstanten der Enzymreaktion wurde gemessen.

Bindungsstudien von Hühnersulfioxidase und Pferdecytochrom c zeigen zwei identische, nicht interagierende Bindungsstellen pro Enzymmonomer. Cytochrom c wird von Sulfioxidase schwächer gebunden als von seinen andern physiologischen Reaktionspartnern. Die Bedeutung der zwei Bindungsstellen pro Enzymmonomer ist unbekannt.

Carbodiimide sind geeignete Reagenzien, um die Bedeutung von Carboxylgruppen für die Aktivität von Sulfitoxidase zu bestimmen, da sie selektiv die Elektronenübertragung von Sulfit zum physiologischen Elektronenakzeptor Cytochrom c hemmen. Die Reaktion mit dem künstlichen Elektronenakzeptor Ferricyanid wird hingegen nicht beeinflusst. Daraus folgt, dass durch die chemische Modifikation Reaktionsschritte beeinflusst werden, die der Oxidation von Sulfit am Molybdän-Zentrum folgen. Durch die Anwesenheit von Cytochrom c im Modifikationsansatz wird die Inaktivierung nicht beeinflusst. Dieses Resultat deutet darauf hin, dass der entscheidende Rest nicht an oder nahe bei der Bindungsstelle von Cytochrom c liegt.

Die Inaktivierung von Sulfitoxidase mit Carbodiimid ist eine Reaktion erster Ordnung. Dieser Befund deutet darauf hin, dass die Modifikation einer oder möglicherweise zweier Carboxylgruppen für die Aktivitätsabnahme verantwortlich ist. Durch die Verwendung von radioaktivem Reagens konnte gezeigt werden, dass die Modifikation von einem oder höchstens zwei Resten für die Inaktivierung verantwortlich ist. Der Rest scheint im Molybdänteil des Enzyms abseits der Cytochrom c Bindungsstelle zu liegen.

Dieser Befund ist sehr erstaunlich. Bei allen bis jetzt untersuchten Reaktionspartnern von Cytochrom c wurde eine Gruppe von richtig platzierten Carboxylresten gefunden, die für die Bindung verantwortlich sind. Keiner dieser Reste ist im strengen Sinn essentiell. Die wichtige Carboxylgruppe der Sulfitoxidase scheint eine andere, direktere Funktion zu haben.

Durch die Modifikation von Imidazolresten mit Diethylpyrocarbonat wird selektiv die Reaktion mit Cytochrom c beeinträchtigt. Die Modifikation eines einzelnen Restes ist für die Inaktivierung verantwortlich. Die Anwesenheit von Cytochrom c im Modifikationsansatz hat keinen Einfluss auf die Inaktivierung. Der essentielle Rest scheint daher ausserhalb der Bindungsstelle für Cytochrom c auf dem Hämtail der Sulfitoxidase zu liegen. Diese Resultate stimmen mit den für Cytochrom c Peroxidase (Bosshard et al., 1984) gefundenen überein. Sie deuten auf eine ähnliche Aufgabe des essentiellen Restes in beiden Proteinen hin, obwohl diese von der Struktur her nicht verwandt sind.

CURRICULUM VITAE

Martin Ritzmann

Bürger von Osterfingen (SH)

geboren in Schaffhausen, am 31. Oktober 1958

Primar- und Sekundarschule in Wilchingen (SH)

1974 - 1978 Kantonsschule Schaffhausen

1978 Matura, Typus C

1978 - 1983 Biochemiestudium an der Phil. Fakultät II der Universität Zürich

1983 Diplom in Biochemie

1983 - 1987 Doktorarbeit am Biochemischen Institut der Universität Zürich
unter der Leitung von Herrn Prof. Dr. H.R. Bosshard

